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PROVISIONAL SPECIFICATION

for the invention entitled:

"A therapeutic agent"

The invention is described in the following statement:

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A THERAPEUTIC AGENT

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to a therapeutic agent. More particularly, the present invention provides a therapeutic agent in the form of a microorganism which is substantially attenuated and has a substantially reduced capacity to grow and replicate due to microbiostatic agents present in, or introduced to, an environment within the host to which the microorganism migrated to following administration, but which is substantially capable of eliciting a humoral and/or cell-mediated immune response to cell surface antigens or antigens secreted, or released, from the microbial cell. Antigens contemplated by the present invention include antigens naturally occurring on, or secreted from, the microbial cell as well as antigens produced through recombinant means such as antigens from other microorganisms, viruses, and parasites. Even more particularly, the therapeutic agent is a species of *Salmonella* or related organism. The therapeutic agent provided by the present invention is useful, *inter alia*, for the prophylaxis, amelioration or treatment of a range of diseases and conditions due to bacterial, viral, fungal and parasitic infections.

DESCRIPTION OF THE PRIOR ART

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Bibliographic details of references provided in this document are listed at the end of the specification.

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Since their development, vaccines have successfully resulted in the eradication of major diseases such as smallpox and have lead to a dramatic reduction in other diseases such as poliomyelitis, hepatitis, measles and tetanus.

An immune response to a foreign antigen generally comprises two mechanisms. A humoral response, which is largely an antibody response directed to antigens present on pathogens in body fluids and a cell-mediated response which is largely a cell based response directed to pathogens which have infected host cells. Activation of the immune system leads to the creation of memory cells that can recognize and repel the same antigens if these reappear in the body.

Vaccines function by eliciting an immune response. However, vaccines vary in the kind and duration of immune protection they can provide. Those based on inactivated or "killed"antigens generally elicit a humoral response only. Such responses are ineffective against pathogens which infiltrate host cells and generally their protection wears off over time which necessitates "booster" vaccinations. Live attenuated vaccines, on the other hand, also have the capacity to elicit a cell-mediated immune response and generally the immunity they provide lasts for the life of the vaccinated subject.

One disease which has yet to be effectively controlled on a global scale is salmonellosis. Salmonellosis is a complex zoonotic disease in terms of its epidemiology, pathogenesis and control. It is a disease arising from infection by certain serotypes of *Salmonella*. In humans, salmonellosis may present clinically as a variety of conditions including gastroenteritis, enteric fever, bacteraemia and focal disease.

Although some advances have been made in strategies and techniques for its control in both humans and animals, salmonellosis still constitutes a major problem in both developed and developing countries (Gomez *et al.*, *World Health Statistics Quarterly 50*:81-89, 1997; World Health Organization, *Salmonellosis control: the role of animal and product hygiene* pp7-78, World Health Organization, Geneva, 1988).

Salmonella enterica subspecies *enterica* serotype Dublin (*S. dublin*) is one example of a microorganism which can cause salmonellosis. *Salmonella dublin* is a host-adapted bacterium which mainly colonizes cattle and calves. Major symptoms of *S. dublin* infection are enteritis and septicaemia in calves, enteritis in adult cattle and abortion in pregnant animals (Field, *Veterinary Journal 104*:251-266, 294-302, 323-339, 1948; Gibson, *Veterinary Record 73*:1284-1295, 1961; Hinton, *British Veterinary Journal 130*:556-562, 1974; Wray and Sojka, *Journal of Dairy Research 44*:383-425, 1977). One of the most important features of *S. dublin* infection is the development of a prolonged carrier state with resultant shedding of bacteria into the environment. The carrier state is unaffected by antimicrobial therapy and bacterial shedding may continue for several years and even throughout the life of the carrier (Vandergraff and Malmo, *Australian Veterinary Journal* 53:453-455, 1977; Wray, *Veterinary Record* 116:485-489, 1985; Wray, *Irish Veterinary Journal* 46:137-140, 1993). This organism, therefore, often becomes endemic to some regions.

Salmonella dublin infection in cattle has been reported in numerous countries around the world, particularly the United Kingdom (UK), Ireland, the United States of America (USA), Australia and many continental European nations (Gibson, *Journal of Dairy Research 32*:97-134, 1965; Wray and Sojka, 1977, *Supra*; Taylor *et al.*, *Journal of Infectious Diseases 146*:322-327, 1982; Bruner, *Cornell Veterinarian 75*:93-96, 1985; Wray, 1985, *Supra*; Wray, 1993, *Supra*; Imberechts, *Salmonella serotypes analysed at the VAR in 2000* pp4-27, Veterinary and Agrochemical Research Centre, Brussels, Belgium, 2001; Murray *et al.*, *Australian Salmonella Reference Centre Annual Report 2000* p4, Institute of Medical and Veterinary Science, Adelaide, Australia, 2001).

Whilst *S. dublin* rarely results in salmonellosis in human subjects, infections that do occur are generally more severe than with other *Salmonella* serovars, and the disease is often fatal (The Centres for Disease Control and Prevention, *MMWR 43*:1-7, 1994). In addition the latter report also states that the major cause of human *S. dublin* infection in the USA was the consumption of contaminated certified raw milk. *Salmonella dublin* is, therefore, an important food borne pathogen. Furthermore, increases in the use of antimicrobial

agents in dairy and beef cattle over the last 50 years have caused the emergence of multiple antimicrobial agent resistant strains in some *Salmonella* serovars, such as *Salmonella enterica* subspecies *enterica* serotype Typhimurium (*S. typhimurium*) DT104. Preventing salmonellosis by vaccination should, therefore, reduce the risk of such strains emerging in the future, making the development of new improved *Salmonella* vaccines a priority.

Vaccines against *S. dublin* infection have been produced using both killed and live *Salmonella*. Killed vaccines are commercially available in some countries to prevent salmonellosis caused by *S. dublin* and *S. typhimurium* in calves and adult cattle. However, although the use of *S. dublin* killed vaccines is reasonably safe, the protection they offer is generally modest (House and Smith, *USAHA Proceedings: evaluation of bovine Salmonella vaccines* United States Animal Health Association, USA, 1997). Live *S. dublin* vaccines, on the other hand, confer better protection to vaccinated animals by inducing a greater cell-mediated immunity (Smith *et al., American Journal of Veterinary Research 45*:2231-2235, 1984). This is believed to be particularly important in the inactivation of facultative intracellular organisms such as *Salmonella* (Lindberg and Robertsson, *Infection and Immunity 41*:751-757, 1983). It is, however, difficult to produce prolonged cell-mediated immunity because this type of immunity is usually active for only a limited time span (Smith, 1984, *Supra*). Moreover, live *Salmonella* vaccines may be potentially pathogenic when administered to animals in poor health or to pregnant animals. For these and other reasons, use of *S. dublin* live vaccines is often restricted (World Health Organization, 1988, *Supra*).

In work leading up to the present invetion, the inventors reasonsed that since attenuated strains of *salmonella* can function as efficient tools for inducing immunity against salmonellosis, they may also act as potential carriers for the expression and delivery of heterologous antigens to the immune system.

Accordingly, the present invention provides a microorganism with the capability to function, *inter alia*, as a live attenuated vaccine that is both effective and safe to use.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Nucleotide and amino acid sequences are referred to by sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided at the end of the specification.

Abbreviations used herein are defined in Table 2.

The present invention provides a therapeutic agent. More particularly, the present invention provides, in one embodiment, a therapeutic agent comprising a microorganism which is substantially attenuated and has a substantially reduced capacity to grow and replicate due to a microbiostatic agent present in, or introduced to, an environment within the host to which it migrates to following administration, wherein said microorganism is capable of eliciting an immune response in a subject, which immune response is directed against an antigen on, or secreted by, the microorganism.

The preferred subjects to which the therapeutic agent of the present invention is administered are livestock species such as cattle, sheep and pigs as well as humans.

The present invention is predicated in part on the determination that microorganisms can be selected according to their inability to grow and replicate in the presence of a microbiostatic agent. This facilitates the selection of a substantially attenuated microorganism which can carry an antigen to the immune system of a host such that it can elicit an immune response.

The ability to select for a substantially attenuated microorganism which can elicit an immune response in a host allows their use as a therapeutic agent in the treatment of disease. The therapeutic agent provided by the present invention is useful, *inter alia*, for the prophylaxis, amelioration or treatment of a range of diseases and conditions, due to bacterial, viral, fungal and parasitic infections.

Any microorganism may be used as the therapeutic agent of the present invention. Preferred microorganisms of the present invention are serotypes of *Salmonella*. Most preferably, the microorganisms of the present invention are of the *S. dublin* serotype.

The selection of a microorganism that is substantially incapable of growth and multiplication in the presence of a microbiostatic agent may be performed using any microbiostatic agent that will not harm the host. The preferred microbiostatic agent of the present invention is bile.

In yet another embodiment, the present invention provides a therapeutic agent comprising a modified microorganism which is substantially attenuated and has a substantially reduced capacity to grow and replicate due to a microbiostatic agent present in, or introduced to, an environment within the host to which it migrates to following administration, wherein said modified microorganism comprises and/or expresses heterologous DNA sequences.

TABLE 1 : SUMMARY OF SEQUENCE IDENTIFIERS

SEQUENCE ID NO.	DESCRIPTION
1	SALGYRA-F
2	SALGYRA-R
3	SALRPOF3
4	RPOBREV

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TABLE 2 : ABBREVIATIONS

ABBREVIATION	DESCRIPTION
AGRF	Australian Genome Research Facility
AR	Analytical regent
Arg	Arginine
Asp	Aspartic acid
BHI	Brain-heart infusion
BDNF	Brain-derived neurotrophic factor
bp	Base pair(s)
BS	Bile salt(s)
B.V.	Booster vaccination
cfu	Colony forming unit(s)
CLED	Cystine-Lactose-Electrolyte Deficient
CM	Cooked meat
CNTF	Cilliary-derived neurotrophic factor
Cys	Cysteine
DDW	Double distilled water
DI	Direct isolation
DNA	Deoxyribonucleic acid
dNTP	dinucleotide triphosphate
EGF	Epidermal growth factor
EI	Enrichment isolation
EPO	Erythropoitin

FGF	Fibroblast grwoth factor
G-CSF	Granulocyte colony stimulating factor
GH	Growth hormone
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GM-CSF	Granulocyte macrophage colony stimulating factor
h	Hours
HE	Hematoxylin and eosin
ID_{50}	50% infectious dose
IFN	Interferon
IL	Interleukin
Ile	Isoleucine
KDa	Kilo-dalton
LD	Lysin decarboxylase
LD_{50}	50% lethal dose
Leu	Leucine
LIF	Leukemia inhibitory factor
LP	Lamina propria
LPS	Lipopolysaccharide
LR	Laboratory reagent
M-CSF	Monocyte colony stimulating factor
MBC	Minimum bactericidal concentration
MCA	MacConkey agar No. 3

MCP	Monocyte chemotactic protein
Met	Methionine
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
min	Minute(s)
MIP	Macrophage inflammatory protein
MLN	Mesenteric lymph node
MN	Mononuclear
MSB	Mannitol selenite broth
NCCLS	The National Committee on Clinical Laboratory Standards
N-R	Resistant to nalidixic acid first and to rifampicin second
n	Number(s)
NGF	Nerve growth factor
NT	Neurotrophin
OD	Optical density
ONPG	O-Nitrophenyl-β-D-galactopyranoside
OSM	Oncostatin M
PBP	Platelet basic protein
PBS	Phosphate buffered saline
PBSF	Pre-B-cell growth stimulating factor
P.B.V.	Post booster vaccination
P.C.	Post challenge
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor

PF	Platelet factor
Phe	Phenylalanine
P.I.	Post inoculation
PMN	Polymorphonuclear neutrophil granulocyte
PMSF	phenyl methyl-sulfonyl fluoride
pmol	Pico mole
Pro	Proline
P.V.	Post vaccination
QRDR	Quinolone-resistance determining region
R-N	Resistant to rifampicin first and to nalidixic acid second
RANTES	Regulated upon activation, normal T-cell expressed
RNA	Ribonucleic acid
RNAi	RNA interference
SBA	10% Sheep blood agar
SCF	Sertoli cell factor
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	Second(s)
Ser	Serine
siRNA	Small interfering RNA
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TGF	Tranforming growth factor
Th cell	Helper T cell

TNF	Tumour necrosis factor
TPO	Thrombopoietin
TSB	Tryptone soya broth
TSI	Triple sugar iron
Tyr	Tyrosine
U	Unit
Val	Valine
VEGF	Vascular endothelial growth factor
XLD	Xylose lysine desoxycholate
°C	Degree Celsius

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatical representation of the isolation and identification of *Salmonella*.

Figure 2 is a graphical representation of growth curves for FD436 and nalidixic acid resistant strains N1 and N6 in TSB media determined by Bioscreen measurements of culture optical density ($OD_{420-580}$) taken every 10 min for 18 h.

Figure 3 is a graphical representation of growth curves for FD436 and rifampicin resistant strain R3 in TSB media determined by Bioscreen measurements of culture optical density ($OD_{420-580}$) taken every 10 min for 18 h.

Figure 4 is a graphical representation of growth curves for FD436 and N-R double antibiotic-resistant strains in TSB media determined by Bioscreen measurements of culture optical density ($OD_{420-580}$) taken every 10 min for 18 h.

Figure 5 is a graphical representation of growth curves for FD436 and R-N double antibiotic-resistant strains in TSB media determined by Bioscreen measurements of culture optical density ($OD_{420-580}$) taken every 10 min for 18 h.

Figure 6 is a photographic representation of agarose gel electrophoresis of amplified PCR products of S. *dublin gyrA*, the sizes of which were later confirmed by sequencing as 347 bp (arrow). Lanes: 1 - 1k bp DNA marker; 2 - *S. dublin* FD436; 3 - N-RM4; 4 - N-RM8; 5 - N-RM9; 6 - N-RM15; 7 - N-RM20; 8 - N-RM25; 9 - N-RM27; 10 - R-NM29.

Figure 7 is a photographic representation of agarose gel electrophoresis of amplified PCR products of *S. dublin rpoB*, the sizes of which were later confirmed by sequencing as between 696 and 714 bp (arrow). Lanes: 1 - 1k bp DNA marker; 2 - *S. dublin* FD436; 3 - N-RM4; 4 - N-RM8; 5 - N-RM9; 6 - N-RM15; 7 - N-RM20; 8 - N-RM25; 9 - N-RM27; 10 - R-NM29.

Figure 8 is a photographic representation of colonies of *S. dublin* wild strain FD436 (A) and mutant N-RM25 (B) growing on SBA plate after aerobic incubation at 37°C for 24 hours. FD436 produced colonies that were white-grey, opaque, circular and convex, and that had an entire margin and a smooth surface. Compared with this, colonies of N-RM25 were smaller, translucent and slightly raised, and had a matte surface.

Figure 9 is a photographic representation of lipopolysaccharide profiles of wild strain FD436 and metabolic-drift mutants determined by SDS-PAGE and ammoniacal silver staining (molecular weights are based on the migration of the protein marker). Lanes: 1 - wild strain FD436; 2 - mutant N-RM4; 3 - N-RM8; 4 - N-RM9; 5 - N-RM15; 6 - N-RM20; 7 - N-RM25; 8 - N-RM27; 9 - R-NM29. Red arrows indicate bands.

Figure 10 is a photographic representation of whole cell protein profiles of wild strain FD436 and metabolic-drift mutants determined by SDS-PAGE and Coomassie blue staining. Lanes: 1 - broad range molecular weight standards; 2 - wild strain FD436; 3 - mutant N-RM4; 4 - N-RM8; 5 - N-RM9; 6 - N-RM15; 7 - N-RM20; 8 - N-RM25; 9 - N-RM27; 10 - R-NM29. Black arrow = band of approximately 53 kDa; Red arrow = band of approximately 24 kDa.

Figure 11 is a graphical representation of growth curves of *S. dublin* wild strain FD436 and metabolic-drift mutants N-RM4 and 25 and R-NM29 in TSB containing graded concentrations of bile salts No. 3 determined by Bioscreen measurements of culture optical density ($OD_{420-580}$) taken every 10 min for 20 h.

Figure 12 is a graphical representation of enumeration of *Salmonella* in liver plus spleen of mice 0 – 24 days following intraperitoneal inoculation with FD436, N-RM4, N-RM25 or R-NM29.

Figure 13 is a photographic representation of a mouse euthanased on Day 7 following challenge with *S. dublin* metabolic-drift mutant N-RM4 (Group 2). Prominent splenomegaly (blue arrow) and grey-white foci (black arrows) scattered across the entire

surface of liver (A) and an enlargement (B) of part of the liver in (A) showing scattered foci (arrows).

Figure 14 is a photographic representation of a mouse euthanased on Day 12 following challenge with *S. dublin* metabolic-drift mutant N-RM25 (Group 3). No gross lesions are evident.

Figure 15 is a photographic representation of acute focal hepatitis. (A) Mouse euthanased on Day 3 following challenge with *S. dublin* wild strain FD436 (Group 1). Prominent PMN infiltration and a suppurative focus (arrow). ×200, H&E stain. (B) Coagulative necrosis (arrow). ×400, H&E stain.

Figure 16 is a photographic representation of penetration by *Salmonella* of liver of mice euthanased on Day 5 following challenge. Organisms were observed in Kupffer cells (black arrows) and PMNs (red arrow). (A) mouse given *S. dublin* wild strain FD436 (Group 1) and (B) mouse given *S. dublin* metabolic-drift mutant N-RM4 (Group 2). ×1000, Giemsa stain.

Figure 17 is a photographic representation of periportal inflammatory infiltrate in liver. Mouse euthanased on Day 3 (A) and Day 24 (B) following challenge with *S. dublin* metabolic-drift mutant N-RM25 (Group 3). Note infiltrating cells are PMNs, lymphocytes and macrophages (A), and lymphocytes and plasma cells (B). ×200, H&E stain.

Figure 18 is a photographic representation of clustered PMNs (arrow) in splenic sinus areas. Mouse euthanased on Day 3 following challenge with *S. dublin* metabolic-drift mutant N-RM4 (Group 2). ×400, H&E stain.

Figure 19 is a photographic representation of the spleen of mouse euthanased on Day 24 following challenge with *S. dublin* metabolic-drift mutant N-RM25 (Group 3). Although there is moderate lymphoid hyperplasia, inflammation is not evident. ×200, H&E stain.

Figure 20 is a photographic representation of the gall bladder from negative control mouse (Group 5). BB = brush border, EP = epithelium, LP = lamina propria, SM = submucosa, MW = muscular wall and S = serosa. ×400, H&E stain.

Figure 21 is a photographic representation of cholecystitis. Mouse euthanased on Day 5 following challenge with *S. dublin* wild strain FD436 (Group 1). Epithelial hyperplasia and oedema of lamina propria are evident. Infiltration of lamina propria by MN cells and a small number of PMNs is also observed. ×400, H&E stain.

Figure 22 is a photographic representation of penetration of gall bladder by *Salmonella*. Mouse euthanased on Day 5 following challenge with *S. dublin* wild strain FD436 (Group 1). The organisms are in the lumen (A) and epithelial cells (B - red arrows). Inflammatory infiltration by PMNs is evident. ×1000, Giemsa stain.

Figure 23 is a photographic representation of severe acute cholecystitis. Mouse euthanased on Day 7 following challenge with *S. dublin* metabolic-drift mutant N-RM4 (Group 2). Epithelial hyperplasia, and oedema, haemorrhage and fibroplasia in lamina propria are prominent. Predominant infiltrating cells are PMNs. Subserosal oedema, fibroplasia and inflammatory infiltrate are also evident (A). ×200, H&E stain. Intraluminal inflammatory exudate of PMNs and MN cells associated with acute cholecystitis in the same mouse (B). ×400, H&E stain.

Figure 24 is a photographic representation of penetration of gall bladder by *Salmonella*. Mouse euthanased on Day 7 following challenge with *S. dublin* metabolic-drift mutant N-RM4 (Group 2). (A) numerous organisms are present in the lumen and adhering to brush border (red arrows). (B) organisms are adhering to epithelial cells (red arrows). Some organisms are in epithelial cells (white arrow). (C) organisms are in epithelial cells (black arrows). Infiltration by PMNs is prominent. ×1000, Giemsa stain.

Figure 25 is a photographic representation of the gall bladder of mouse euthanased on Day 5 following challenge with *S. dublin* metabolic-drift mutant N-RM25 (Group 3). (A) mild

epithelial hyperplasia, congestion of lamina propria, margination in vessels (arrows) and mild infiltration suggesting early stage inflammation. ×400, H&E stain. (B) *Salmonella* are present in gall bladder lumen in low numbers (arrows). ×1000, Giemsa stain.

Figure 26 is a photographic representation of the gall bladder of mouse euthanased on Day 24 following challenge with *S. dublin* metabolic-drift mutant N-RM25 (Group 3). Inflammation is not evident except very mild oedema of lamina propria and subserosa. ×400, H&E stain.

Figure 27 is a graphical representation of the number of *Salmonella* in liver and spleen of mice (n = 5/group) dosed using single and double vaccination methods with different doses of N-RM25 and challenged with wild strain FD436.

Figure 28 is a graphical representation of the number of *Salmonella* in liver and spleen of mice vaccinated with N-RM4 or R-NM29 and challenged with wild strain FD436.

DETAILED DESCRIPTION OF THE INVENTION

The present invention contemplates a therapeutic agent in the form of a microorganism which is substantially attenuated and has a substantially reduced capacity to grow and replicate due to microbiostatic agents present in, or introduced to, an environment within the host to which the microorganism migrates following administration. This facilitates the selection of a substantially attenuated microorganism which can carry an antigen to the immune system of a host such that it can elicit an immune response. The therapeutic agent provided by the present invention is useful, *inter alia*, for the prophylaxis of a range of diseases or conditions, and in particular, diseases or conditions which can be treated, or symptoms ameliorated by, the elicitation of an immune response. Such diseases and conditions include, *inter alia*, infection by bacteria, viruses, fungi and parisites.

Prior to describing the present invention in detail, it is to be understood that unless otherwise indicated, the subject invention is not limited to specific therapeutic components, manufacturing methods, dosage regimens, or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must also be noted that, as used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to a "therapeutic agent" includes a single therapeutic agent, as well as two or more therapeutic agents; reference to a "microorganism" includes a single microorganism, as well as two or more microorganisms; and so forth.

In one embodiment the present invention provides a therapeutic agent comprising a microorganism which is substantially attenuated and has a substantially reduced capacity to grow and replicate due to a microbiostatic agent present in, or introduced to, an environmentwithin the host to which it migrates following administration, wherein said microorganism is capable of eliciting an immune response in a subject, which immune response is directed against an antigen produced by said microorganism.

Subject as used herein refers to humans and non-human primates (*e.g.* gorilla, macaque, marmoset), livestock animals (*e.g.* sheep, cow, horse, donkey, pig), companion animals (*e.g.* dog, cat), laboratory test animals (*e.g.* mouse, rabbit, rat, guinea pig, hamster), captive wild animals (*e.g.* fox, deer) and any other organisms who can benefit from the therapeutic agent of the present invention. There is no limitation on the type of animal that could benefit from the presently described therapeutic agents. The most preferred subjects of the present invention are livestock animals and humans. A subject regardless of whether it is a human or non-human may be referred to as a patient, subject, individual, animal, host or recipient.

Reference herein to "microorganism" means any prokaryotic organism (*e.g.* bacteria) or lower eukaryotic organism (*e.g.* algae, fungi, protozoa). The preferred microorganisms of the present invention are members of the Enterobacteriaceae group of bacteria. The Enterobacteriaceae group comprises, but is not limited to, *Enterobacter* (*e.g. Enterobacter aerogenes, Enterobacter amnigenus, Enterobacter asburiae, Enterobacter cancerogenus, Enterobacter cloacae, Enterobacter cowanii, Enterobacter dissolvens, Enterobacter gergoviae, Enterobacter hormaechei, Enterobacter intermedius, Enterobacter kobei, Enterobacter nimipressuralis, Enterobacter pyrinus, Enterobacter sakazakii, Enterobacter sp., Enterobacter sp. 'MS 412', Enterobacter sp. 16-31, Enterobacter sp. 2002-2301161, Enterobacter sp. 22, Enterobacter sp. 253a, Enterobacter sp. 3-45, Enterobacter sp. 76996, Enterobacter sp. B2/69, Enterobacter sp. B24a, Enterobacter sp. B24b, Enterobacter sp. B41, Enterobacter sp. B509, Enterobacter sp. B5R5, Enterobacter sp. B901-2, Enterobacter sp. B96, Enterobacter sp. CIT5, Enterobacter sp. CC1, Enterobacter sp. CH2-4, Enterobacter sp. dtb33, Enterobacter sp. DW143a1, Enterobacter sp. DW56, Enterobacter sp. EK3.1, Enterobacter sp. EK4, Enterobacter sp. Fma, Enterobacter sp. IMD1260, Enterobacter sp. isolate #3, Enterobacter sp. Lgg10.16, Enterobacter sp. Lgg10.2, Enterobacter sp. Lgg5.6, Enterobacter sp. NAB4, Enterobacter sp. NAB5, Enterobacter sp. PD12, Enterobacter sp. RFL1396, Enterobacter sp. S23, Enterobacter sp. S4-27, Enterobacter sp. TUT1014*), *Erwinia* (*e.g. Candidatus Erwinia dacicola, Erwinia amylovora, Erwinia aphidicola, Erwinia billingiae, Erwinia*

chrysantum, Erwinia mallotivora, Erwinia papayae, Erwinia persicina, Erwinia psidii, Erwinia pyrifoliae, Erwinia rhapontici, Erwinia tracheiphila, Erwinia sp., Erwinia sp. 1, Erwinia sp. 2, Erwinia sp. 3, Erwinia sp. 4, Erwinia sp. 5, Erwinia sp. 6, Erwinia sp. DW1, Erwinia sp. Ejp 556, Erwinia sp. Ejp557, Erwinia sp. Ejp617, Erwinia sp. Lgg10.4, Erwinia sp. Lgg20.9, Erwinia sp. MB31, Erwinia sp. P18G1186, Erwinia sp. SK-30-7, Erwinia sp. TzT-JG-2-8), Escherichia (e.g. Escherichia albertii, Escherichia blattae, Escherichia coli, Escherichia coli 042, Escherichia coli B, Escherichia coli CFT073, Escherichia coli E2348, Escherichia coli K12, Escherichia coli O111:H-, Escherichia coli O127:H6, Escherichia coli O157:H-, Escherichia coli O157:H7, Escherichia coli O157:H7 EDL933, Escherichia coli O6, Escherichia fergusonii, Escherichia hermannii, Escherichia senegalensis, Escherichia vulneris, Escherichia sp. 253b, Escherichia sp. Souza-207, Escherichia sp. Souza-273, Escherichia sp. Souza-57), Klebsiella (e.g. Klebsiella aerogenes, Klebsiella granulomatis, Klebsiella milletis, Klebsiella oxytoca, Klebsiella cf. planticola B43, Klebsiella pneumoniae, Klebsiella pneumoniae subsp. ozaenae, Klebsiella pneumoniae subsp. pneumoniae, Klebsiella pneumoniae subsp. rhinoscleromatis, Klebsiella senegalensis, Klebsiella singaporensis, Klebsiella variicola, Klebsiella sp., Klebsiella sp. 141, Klebsiella sp. 6613, Klebsiella sp. 6A2, Klebsiella sp. 708, Klebsiella sp. 910, Klebsiella sp. A5054, Klebsiella sp. ABR11, Klebsiella sp. B2-1, Klebsiella sp. CC-88028, Klebsiella sp. CC-88037, Klebsiella sp. CC-88168, Klebsiella sp. Cd-1, Klebsiella sp. Ck-1, Klebsiella sp. DW40, Klebsiella sp. ES14.2, Klebsiella sp. HL1, Klebsiella sp. K2-1, Klebsiella sp. KCL-2, Klebsiella sp. KCL2, Klebsiella sp. KG1, Klebsiella sp. KGA, Klebsiella sp. LS13-39, Klebsiella sp. LX3, Klebsiella sp. M4112, Klebsiella sp. Ni3, Klebsiella sp. P2, Klebsiella sp. P3, Klebsiella sp. P4, Klebsiella sp. PN2, Klebsiella sp. rennanqilfy18, Klebsiella sp. RS1, Klebsiella sp. TK44, Klebsiella sp. VI), Proteus (e.g. Proteus inconstans, Proteus mirabilis, Proteus penneri, Proteus vulgaris, Proteus sp. LE138), Salmonella (e.g. Salmonella bongori, Salmonella bongori 12149, Salmonella bongori 66:z41:-, Salmonella choleraesuis, Salmonella choleraesuis subsp. arizonae, Salmonella choleraesuis subsp. choleraesuis, Salmonella choleraesuis subsp. diarizonae, Salmonella choleraesuis subsp. houtenae, Salmonella choleraesuis subsp. indica, Salmonella choleraesuis subsp. salamae, Salmonella enterica, Salmonella enterica subsp. arizonae, Salmonella enterica subsp. diarizonae, Salmonella enterica IIIb 50:k:z,

Salmonella enterica subsp. enterica, Salmonella enterica subsp. enterica serovar 1,4,[5],12,:1:1,2, Salmonella enterica subsp. enterica serovar Abony, Salmonella enterica subsp. enterica serovar Abortusequi, Salmonella enterica subsp. enterica serovar Abortusovis, Salmonella enterica subsp. enterica serovar Adelaide, Salmonella enterica subsp. enterica serovar Agona, Salmonella enterica subsp. enterica serovar Albany, Salmonella enterica subsp. enterica serovar Anatum, Salmonella enterica subsp. enterica serovar Austin, Salmonella enterica subsp. enterica serovar Azteca, Salmonella enterica subsp. enterica serovar Banana, Salmonella enterica subsp. enterica serovar Bareilly, Salmonella enterica subsp. enterica serovar Berta, Salmonella enterica subsp. enterica serovar Blockley, Salmonella enterica subsp. enterica serovar Borreze, Salmonella enterica subsp. enterica serovar Bovis-morbificans, Salmonella enterica subsp. enterica serovar Braenderup, Salmonella enterica subsp. enterica serovar Brandenburg, Salmonella enterica subsp. enterica serovar Bredeney, Salmonella enterica subsp. enterica serovar Budapest, Salmonella enterica subsp. enterica serovar Bury, Salmonella enterica subsp. enterica serovar California, Salmonella enterica subsp. enterica serovar Chester, Salmonella enterica subsp. enterica serovar Chingola, Salmonella enterica subsp. enterica serovar Choleraesuis, Salmonella enterica subsp. enterica serovar Cubana, Salmonella enterica subsp. enterica serovar Derby, Salmonella enterica subsp. enterica serovar Dublin, Salmonella enterica subsp. enterica serovar Enteritidis, Salmonella enterica subsp. enterica serovar Essen, Salmonella enterica subsp. enterica serovar Gallinarum, Salmonella enterica subsp. enterica serovar Gallinarum/pullorum, Salmonella enterica subsp. enterica serovar Give, Salmonella enterica subsp. enterica serovar Hadar, Salmonella enterica subsp. enterica serovar Haifa, Salmonella enterica subsp. enterica serovar Havana, Salmonella enterica subsp. enterica serovar Heidelberg, Salmonella enterica subsp. enterica serovar Infantis, Salmonella enterica subsp. enterica serovar Java, Salmonella enterica subsp. enterica serovar Kentucky, Salmonella enterica subsp. enterica serovar Kottbus, Salmonella enterica subsp. enterica serovar Liverpool, Salmonella enterica subsp. enterica serovar London, Salmonella enterica subsp. enterica serovar Maregrosso, Salmonella enterica subsp. enterica serovar Marrtens, Salmonella enterica subsp. enterica serovar Matopeni, Salmonella enterica subsp. enterica serovar Mbandaka, Salmonella enterica subsp. enterica serovar Mikawasima, Salmonella enterica

subsp. enterica serovar Minnesota, Salmonella enterica subsp. enterica serovar Monschaut, Salmonella enterica subsp. enterica serovar Montevideo, Salmonella enterica subsp. enterica serovar Moscow, Salmonella enterica subsp. enterica serovar Muenchen, Salmonella enterica subsp. enterica serovar Muenster, Salmonella enterica subsp. enterica serovar Naestved, Salmonella enterica subsp. enterica serovar Newmexico, Salmonella enterica subsp. enterica serovar Newport, Salmonella enterica subsp. enterica serovar Ohio, Salmonella enterica subsp. enterica serovar Oranienburg, Salmonella enterica subsp. enterica serovar Ordonez, Salmonella enterica subsp. enterica serovar Othmarschen, Salmonella enterica subsp. enterica serovar Panama, Salmonella enterica subsp. enterica serovar Paratyphi B, Salmonella enterica subsp. enterica serovar Paratyphi C, Salmonella enterica subsp. enterica serovar Pensacola, Salmonella enterica subsp. enterica serovar Potsdam, Salmonella enterica subsp. enterica serovar Pullorum, Salmonella enterica subsp. enterica serovar Rachaburi, Salmonella enterica subsp. enterica serovar Reading, Salmonella enterica subsp. enterica serovar Rostock, Salmonella enterica subsp. enterica serovar Rubislaw, Salmonella enterica subsp. enterica serovar Saintpaul, Salmonella enterica subsp. enterica serovar Schleissheim, Salmonella enterica subsp. enterica serovar Senftenberg, Salmonella enterica subsp. enterica serovar Setubal, Salmonella enterica subsp. enterica serovar Shomron, Salmonella enterica subsp. enterica serovar Simsbury, Salmonella enterica subsp. enterica serovar Sloterdijk, Salmonella enterica subsp. enterica serovar Sofia, Salmonella enterica subsp. enterica serovar Stanley, Salmonella enterica subsp. enterica serovar Tejas, Salmonella enterica subsp. enterica serovar Tennessee, Salmonella enterica subsp. enterica serovar Tennyson, Salmonella enterica subsp. enterica serovar Texas, Salmonella enterica subsp. enterica serovar Thompson, Salmonella enterica subsp. enterica serovar Toulon, Salmonella enterica subsp. enterica serovar Travis, Salmonella enterica subsp. enterica serovar Typhi, Salmonella enterica subsp. enterica serovar Typhimurium, Salmonella enterica subsp. enterica serovar Typhisuis, Salmonella enterica subsp. enterica serovar Vellore, Salmonella enterica subsp. enterica serovar Virchow, Salmonella enterica subsp. enterica serovar Virginia, Salmonella enterica subsp. enterica serovar Waycross, Salmonella enterica subsp. enterica serovar Weltevreden, Salmonella enterica subsp. enterica serovar Wien, Salmonella enterica subsp. houtenae, Salmonella enterica IV 43:z4,z23:-,

Salmonella enterica subsp. houtenae serovar Houten, Salmonella enterica subsp. indica, Salmonella enterica VI 1,6,14,25:a:e,n,x, Salmonella enterica subsp. salamae, Salmonella enterica subsp. salamae serovar 4,12,27:i:z35, Salmonella enterica subsp. salamae serovar 4,12:z:1,7, Salmonella enterica subsp. VII, Salmonella enteritidis, Salmonella paratyphi, Salmonella typhi, Salmonella typhimurium, Salmonella typhimurium DT104, Salmonella typhimurium LT2, Salmonella typhimurium SL1344, Salmonella typhimurium TR7095, Salmonella sp., Salmonella sp. 4182, Salmonella sp. AHL 6, Salmonella sp. S126, Salmonella sp. S14, Salmonella sp. S191, Salmonella sp. TC67), Shigella (e.g. Shigella boydii, Shigella dysenteriae, Shigella dysenteriae M131649, Shigella flexneri, Shigella flexneri 2a, Shigella sonnei, Shigella sonnei 53G, Shigella sp.) and *Yersinia (e.g. Yersinia aldovae, Yersinia bercovieri, Yersinia bercovieri (type 0:58,16), Yersinia enterocolitica, Yersinia enterocolitica (type 0:1), Yersinia enterocolitica (type 0:13,18), Yersinia enterocolitica (type 0:13a,13b), Yersinia enterocolitica (type 0:15), Yersinia enterocolitica (type 0:2), Yersinia enterocolitica (type 0:20), Yersinia enterocolitica (type 0:21), Yersinia enterocolitica (type 0:3), Yersinia enterocolitica (type 0:34), Yersinia enterocolitica (type 0:4,32), Yersinia enterocolitica (type 0:5,27), Yersinia enterocolitica (type 0:8), Yersinia enterocolitica (type 0:9), Yersinia frederiksenii, Yersinia intermedia, Yersinia kristensenii, Yersinia mollaretii, Yersinia pestis, Yersinia pestis CO92, Yersinia pestis KIM, Yersinia pseudotuberculosis, Yersinia pseudotuberculosis (type O:1b), Yersinia rohdei, Yersinia ruckeri, Yersinia sp.)*.

Even more preferably, the microorganisms of the present invention are serotypes of *Salmonella*.

Accordingly, another embodiment of the present invention provides a therapeutic agent comprising a serotype of *Samonella* which is substantially attenuated and has a substantially reduced capacity to grow and replicate due to a microbiostatic agent present in, or introduced to, an environment within the host to which it migrates to following administration, wherein said serotype of *Samonella* is capable of eliciting an immune response in a subject, which immune response is directed against an antigen produced by said *Samonella*.

Preferably, the microorganism of the present invention functions as a "carrier". Reference herein to a "carrier" means a microorganism which can carry an antigen (either naturally occurring or heterologous) such that it can elicit a desired biological response in a subject. Preferably the desired biological response is a humoral and/or cell-mediated immune response. Even more preferably, the biological response of the present invention results in the prevention, amelioration or treatment of symptoms associated with a disease or condition such as, but not limited to, salmonellosis.

Most preferably, the microorganism of the present invention is *S. dublin*.

Accordingly another embodiment of the present invention provides a therapeutic agent comprising *S.dublin* which is substantially attenuated and has a substantially reduced capacity to grow and replicate due to a microbiostatic agent present in, or introduced to, an environment within the host to which it migrates to following administration, wherein said *S. dublin* is capable of eliciting an immune response in a subject, which immune response is directed against an antigen produced by said *S. dublin*.

Reference herein to "*S. dublin*" also includes all *S. dublin* variants, such as those *S. dublin* which are genetically modified and *S. dublin* related serovars (*i.e.* other *Salmonella* species or sub-species with related antigenic properties).

The microorganisms contemplated by the present invention are substantially attenuated. Reference herein to "attenuated" means a microorganism that does not induce an infection or symptoms of an infection in a host compared to a non-attenuated organism present in the same amount.

One method of attenuation used in accordance with the present invention is genetic attenuation. Reference herein to "genetic attenuation" means attenuating a microorganism through modification of the microorganism genome using sense, antisense, RNAi, si-RNA or mutation technology. A preferred method of modifying the genome of the

microorganism of the present invention is mutation. This involves exposing nucleic acids which comprise the genome of a microorganism to mutagens such as chemical and radiation mutagens to induce single or multiple nucleotide substitutions, additions, deletions and/or insertions into genes or genetic regions required for, or which, facilitate pathogenesis in or on a host.

In a particularly preferred embodiment of the present invention, the mutation of a microorganism genome is achieved by exposing the microorganism to high concentrations of nalidixic acid and rifampicin. This results in double metabolic-drift mutant microorganisms whose genomes comprise two or more nucleotide mutations.

The substantially attenuated microorganism of the present invention preferably has a substantially reduced capacity to grow and replicate due to a microbiostatic agent present in, or introduced to, an environment to which it is administered.

Reference herein to "substantially reduced capacity to grow and replicate" means that the microorganism is capable of survival and eliciting an immune response without growing and generating substantial numbers of progeny. This may be achieved by any means well known to those in the art. In a preferred embodiment it is achieved by a microbiostatic agent. Reference herein to a "microbiostatic agent" means an agent that will render a microorganism substantially incapable of growth and multiplication but which is not microbicidal at the concentration present. The term "microbiostatic agent" should also be taken to include a reference to an "inhibitory agent" and as such the terms may be used interchangably.

The microorganisms of the present invention are therefore selected according to their inability to grow and multiply in the presence of a microbiostatic agent. The preferred microorganisms of the present invention have a substantially reduced capacity to grow and replicate in the presence of a microbiostatic agent.

Although the microbiostatic agent of the present invention may also comprise any agent which has microbiostatic properties, in a preferred embodiment, the microbiostatic agent of the present invention is naturally present in the environment to which the microorganism of the present invention is administered. Most preferably, the microbiostatic agent of the present invention is bile.

Accordingly, in another embodiment the present invention provides a therapeutic agent comprising *S. dublin* which is substantially attenuated and has a substantially reduced capacity to grow and replicate due to bile salts present in, or introduced to, an environment within the host to which it migrates to following administration, wherein said *S. dublin* is capable of eliciting an immune response in a subject, which immune response is directed against an antigen produced by said *S. dublin*.

Microbiostatic agents can either be naturally present in the environment to which the present invention is administered or may be introduced, either simultaneously or sequentially, with the present invention at the time of administration. Sequential administration includes administration within nanoseconds, seconds, minutes, hours or days. Preferably, within seconds or minutes.

In one embodiment the present invention comprises a microorganism which only expresses naturally occurring antigens. However, the development of recombinant nucleic acid technology has facilitated the ability to modify the microorganisms of the present invention such that they also contain and/or express heterologous nucleic acid sequences.

Heterologus nucleic acid sequences may be introduced into the microorganism such that the nucleic acid remains extrachromosomal. The introduced nucelic acid sequences may also be in the form of artificial chromosomes, such as but not limited to bacterial artificial chromososmes (BACs). Alternatively, heterologus nucleic acid sequences may also incorporated directly into chromosomes naturally present within the microorganism. Methods for nucleic acid transfer to the microorganisms of the present invention are well known in the art (*e.g.* Sambrook and Russell, *Molecular Cloning : A Laboratory Manual*,

3rd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001; Ausubel (Ed), *Current Protocols in Molecular Biology*, 5th Edition, John Wiley & Sons, Inc, NY, 2002) and include, but are not limited to, electroporation and calcium chloride-mediated transfection.

Heterologous nucleic acid sequences introduced to the microorganisms of the present invention facilitate "DNA vaccination" or allow the expression of heterologous proteins which may function, *inter alia*, as antigens or as factors which enhance the immunogenicity of a particular antigen.

The introduced heterologous nucleic acid sequences may co-exist and/or be co-expressed with other nucleic acid sequences already present in the micoorganism of the present invention. This facilitates the co-expression of heterologous nucleic acid sequences, preferably in the form of antigens or factors which enhance the immunogenicity of a particular antigen, with the naturally occuring antigens on the microoganism of the present invention.

Accordingly, in another embodiment the present invention provides a therapeutic agent comprising a modified microorganism containing and/or expressing heterologous nucleic acid sequences which is substantially attenuated and incapable of growth and multiplication due to a microbiostatic agent present in, or introduced to, an environment within the host to which it migrates following administration.

Examples of proteins which may function as antigens include, but are not limited to, cell-surface proteins associated with microrganisms (*e.g.* bacteria, algae, fungi and protozoa) and viruses which cause upper respiratory tract infections, pleuropulmonary and bronchial infections, cardiovascular infections, urinary tract infections, sexually transmitted infections, nervous system infections, skin and soft tissue infections, gastrointestinal infections, bone and joint infections, eye infections and the like.

Examples of factors which may enhance the immunogenicity of a particular antigen include, but are not limited to, Brain-derived neurotrophic factor (BDNF), Cilliary-derived neurotrophic factor (CNTF), Epidermal growth factor (EGF), Erythropoitin (EPO), Fibroblast growth factor (FGF) 1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF7, FGF8, FGF9, FGF10, FGF11, FGF12, FGF12, FGF13, FGF14, FGF15, FGF16, FGF17, FGF18, FGF19, FGF20, FGF21, FGF22, FGF23, Granulocyte colony stimulating factor (G-CSF), Granulocyte macrophage colony stimulating factor (GM-CSF), Interferon (IFN) α, IFNβ, IFNγ, Interleukin (IL) 1, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL11, IL12, IL13, IL14, IL15, Leukemia inhibitory factor (LIF), Monocyte chemotactic protein (MCP) 1, MCP2, MCP3, MCP4, MCP5, Macrophage colony stimulating factor (M-CSF), Macrophage inflammatory protein (MIP) 1, MIP2, Nerve growth factor (NGF), Neurotrophin (NT) 3, NT4, NT5, NT6, NT7, Oncostatin M (OSM), Platelet basic protein (PBP), Pre-B-cell growth stimulating factor (PBSF), Platelet derived growth factor (PDGF), Platelet factor (PF) 4, Regulated upon activation, normal T-cell expressed (RANTES), Sertoli cell factor (SCF), Transforming growth factor (TGF) α, TGFβ, Tumour necrosis factor (TNF) α, TNFβ, Thrombopoietin (TPO), Vascular endothelial growth factor (VEGF), Growth hormone (GH), insulin and the like.

The identification of therapeutic agents in the form of microorganisms capable of eliciting a humoral and/or cell-mediated immune response to cell surface antigens or antigens secreted or released from a cell enables these agents to be used in the prophylaxis and/or treatment of a range of diseases and conditions. Examples of diseases and conditions which may be prevented, ameliorated or treated by the therapeutic agents of the present invention include, but are not limited to, bacterial, viral, fungal and parasitic infections.

Reference herein to "treatment" may mean a reduction in the severity of an existing condition. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Reference herein to the term "prophylaxis" means to prevent the onset of a particular disease or condition. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease or condition.

The therapeutic agent of the present invention may be administered to a subject by any convenient means known to one skilled in the art. Routes of administration may include, but are not limited to, oral, intraperitoneal and parenteral routes.

The amount of therapeutic agent necessary to administer to a subject is an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of a particular disease or condition being treated. The amount varies depending upon the species of the subject to be treated, the health and physical condition of the subject to be treated, the degree of protection desired, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The therapeutic agent of the present invention is generally administered along with a pharmaceutically acceptable carrier or diluent. The particular pharmaceutically acceptable carrier or diluent used is not critical to the present invention. Examples of diluents include phosphate buffered saline, buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 7.0) alone (Levine *et al.*, *Journal of Clinical Investigation 79*:888-902, 1987; Black *et al.*, *Journal of Infectious Diseases 155*:1260-1265, 1987), or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame (Levine *et al.*, *Lancet II*:467-470, 1988). Examples of carriers include proteins such as those as found in skim milk, sugars such as sucrose, or polyvinylpyrrolidone. Typically these carriers would be used at a concentration of about 0.1-90% (w/v) but preferably at a range of 1-10% (w/v).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

The present invention is further described by the following non-limiting examples.

EXAMPLE 1

General experimental procedures

The following experimmetal procedures are used in the subsequent examples which follow:

Materials:

Bacteria:

A *S. dublin* wild strain FD436 was obtained from the Department of Primary Industries' Animal Research Institute (ARI) and was used to generate metabolic-drift mutants. It was also the challenge strain used in mouse inoculation studies. This organism was the causative organism of an outbreak of salmonellosis in Beaudesert, Queensland, Australia in 1998.

Chemicals:

All chemicals were used in accordance with the manufacturer's recommendations. Information in square brackets indicates catalogue numbers of products.

Commercial chemicals:

Ajax Laboratory Chemicals (Ajax), New South Wales (NSW), Australia:

Glycerol

Sodium hydroxide pellets (analytical reagent (AR))

BDH Chemicals (BDH), Victoria, Australia:

Acetic acid

Bromophenol blue

Ethanol

Glycine

Hydrochloric acid (HCl) sp.gr.1.18 (AR)

Methanol 99.8 atom%

Sodium hydrogen carbonate ($NaHCO_3$) (AR)

Boehringer Ingelheim Pty. Ltd., Animal Health Division, NSW, Australia:

Nembutal (Pentobarbitone sodium)

Gibco BRL, USA

Ethylenediaminetetraacetic acid (EDTA)

Sodium dodecyl sulfate (SDS)

Tris

LKB-Produkter, Sweden

Acrylamide

Merck-Schuchardt, Germany

2-mercapto-ethanol

Murex Biotech Ltd. (Murex), Dartford, England:

Salmonella polyvalent agglutinating serum (polyvalent O groups A-S) [ZC02]

Progen Industries Ltd., NSW, Australia

Agarose (DNA grade)

Sigma Chemical Co. (Sigma), St. Louis, Missouri, USA:

Antimicrobials

Nalidixic acid [N-8878]

Rifampicin approximately 95% [R-3501]

Solutions:

Phosphate buffered saline (PBS) ×1:

Sodium Chloride	20 g
Potassium Chloride	0.5 g
Na_2HPO_4	2.29 g
KH_2PO_4	0.5 g
Distilled water	2500 ml

The mixture was sterilized by autoclaving at 121°C, 15 lb for 15 minutes (min).

Equipment:

Labsystems Co., Helsinki, Finland:

Bioscreen analyser system

Media:

In the production of media, any necessary adjustments to pH were made with HCl or NaOH and media were autoclaved at 121°C, 15 lb for 15 min, unless otherwise stated.

Commercial media:

Media were prepared following the manufacturer's recommendations and made up to volume with distilled water.

Oxoid Ltd. (Oxoid), Hampshire, England:

Brain-heart infusion (BHI) broth [CM225]
Cystine-Lactose-Electrolyte Deficient (CLED) medium [CM301]
Lysine decarboxylase (LD) broth (Taylor modification) [CM308]
MacConkey agar No. 3 (MCA) medium [CM1 15]
Mannitol selenite broth (MSB) base [L121]
Mueller-Hinton agar (MHA) medium [CM337]
Triple sugar iron (TSI) agar [CM277]
Tryptone soya broth (TSB) [CM129]

Xylose lysine desoxycholate (XLD) medium [CM469]

Laboratory made media:

Cooked meat (CM) broth:

Beef hearts, from which fat and sinew had been removed, were minced. NaOH of 1/500 of the weight of the mince was dissolved in a volume of water equal to the weight of the mince. The mince was added to the NaOH solution and the mixture boiled for 20 min. After cooling, solid fat in the mixture was removed. The mixture was then filtered through gauze to remove any liquid. The dry mince was packed into 1 oz narrow bottles to about 1/4 full. Bottles were filled with BHI broth and autoclaved.

ONPG broth:

ONPG solution:

O-Nitrophenyl-β-D-galactopyranoside	0.5 g
0.1M potassium phosphate	84.0 ml

The solution was sterilised with a 0.45 U millipore filter and stored at 5°C for up to 2 weeks.

Basal medium:

Tryptone [Oxoid, L42]	1.0 g
Sodium chloride	0.5 g
Distilled water	100.0 ml

The pH of the medium was adjusted to 7.4 and the medium was autoclaved.

Twenty-five millilitres of the ONPG solution was aseptically added to 75 ml of the basal medium. The mixture was dispensed into 1/4 oz bottles in 2 ml amounts and stored at 5°C until use.

Semi-solid medium:

The following ingredients were combined and heated to boiling point whilst stirring then cooled and the pH adjusted to 7.0.

'Lab-Lemco' powder [Oxoid, L29]	1.0 g
Peptone bacteriological [Oxoid, L37]	1.0 g
Sodium chloride	0.5 g
Agar technical [Oxoid, L13]	0.2 g
Distilled water	100 ml

10% sheep blood agar (SBA):

Blood agar base:

Blood agar base No. 2 [Oxoid, CM271]	40 g
Distilled water	1000 ml

The blood agar base was autoclaved and kept at 56°C until ready to pour then cooled to 50°C. Sterile sheep blood was mixed with the blood agar base at a ratio of 1:9. The mixture was poured onto plates and the plates dried in an incubator overnight. The plates were stored at 5°C until use.

Carbohydrate fermentation broth media:

One millilitre of Andrade's indicator was added to 90 ml of 1% peptone water. One gram of each of the following carbohydrates was dissolved in 9 ml of distilled water and then added to the peptone water. These mixtures were then filtered through a millipore filter (0.2 μm). The mixture was dispensed into 1/4 oz bottles in 2 ml amounts and stored at room temperature in a dark room until use.

0.04% (w/v) nalidixic acid and/or 0.02% (w/v) rifampicin SBA:

2% (w/v) nalidixic acid solution:

Nalidixic acid	200 mg
1N NaOH	2 ml
Sterilised distilled water	8 ml

1% (w/v) rifampicin solution:

Rifampicin	100 mg
Methanol (99.8 atom%)	10 ml

The blood agar base was autoclaved and kept at 56°C until ready to pour then cooled to 50°C. Sterile sheep blood was mixed with the blood agar base at a ratio of 1:9. One millilitre of either 2% nalidixic acid or 1% rifampicin solution, or both were then added to a mixture of the blood agar base and the sheep blood to make a total volume of 50 ml. The mixture was poured onto the plates and the plates dried in an incubator overnight. The plates were stored at 5°C until use.

Experimental animals:

Mice:

Unless otherwise stated, conventional female mice (20-25g) were purchased from the UQ Central Animal Breeding House. To minimise the influence of individual variation on experimental results, mice with the same genetic line were used. The mice were housed in standard mice cages (the number of mice in each cage was equal to or less than five) and were given commercial mouse pellets and clean water. Health of the mice was monitored once a day (9:00 AM) for 3 days before commencing experiments. Twenty pieces of faeces in each cage were randomly taken everyday over this period and tested using the *Salmonella* isolation and identification methods to confirm the mice were free from *Salmonella*. The bedding was renewed every day and the cages were sterilised with 70% ethyl alcohol and then flamed at each renewal. Mice showing any clinical signs were isolated from other healthy mice immediately after the finding and replaced by healthy mice.

Methods:

Bacteria Storage:

One colony from a pure culture was inoculated onto a 10% (v/v) SBA plate then incubated at 37°C for 24 hours (h). Bacteria growing on the plate were mixed with BHI broth to obtain a suspension of approximately 10^9 bacteria per ml. Aliquots of 0.5 ml each were then distributed into small vials and stored at −70°C until required, at which time aliquots were cultured once on SBA at 37°C for 24 h and then used for the experiment, unless otherwise stated.

Bacterial enumeration:

The number of viable bacterial cells in a specimen was estimated as cfu using the Miles-Misra surface plate-count method (Miles *et al.*, *Journal of Hygiene Cambridge 38*:732-749, 1938), unless otherwise stated. Ten-fold dilution of specimens containing *Salmonella* were made in PBS. One hundred microliters of each dilution were inoculated onto SBA plates. For estimating the number of nalidixic acid and/or rifampicin resistant mutants, 0.04% (w/v) nalidixic acid and/or 0.02% (w/v) rifampicin SBA plates were also used. These plates were incubated at 37°C for 24 to 48 h before counting the number of colonies on the plates.

Salmonella isolation:

Unless otherwise stated, three methods were employed to isolate *Salmonella* including its metabolic-drift mutants. When the organisms were not isolated through the direct isolation (DI) method, the enrichment isolation (EI) methods were employed (Figure 1).

DI method for all specimens: Specimens were directly plated onto SBA, MCA and XLD plates. These media were then incubated at 37°C for 24 to 48 h.

EI method for blood, abdominal fluid and bile specimens: A specimen was added to 20 ml of CM broth and incubated at 37°C. After 48 h, 50 µl of the broth were plated onto SBA, MCA and XLD plates. These plates were then incubated at 37°C for 24 to 48 h.

EI method for tissue and faecal specimens: Up to 1 ml of homogenate of each sample was added to 10 ml of MSB containing 0.0001 g L-cystine (BDH) and incubated at 37°C for 24 h. Fifty microlitres of MSB were then plated onto SBA, MCA and XLD plates. These plates were incubated at 37°C for 24 to 48 h.

Salmonella identification:

After isolation, three colonies suspected of being *Salmonella* from each XLD plate were transferred to CLED media. After incubation for 48 h, colonies suspected of being *Salmonella* were then tested serologically in the polyvalent O antigen slide agglutination test. Colonies which produced a positive result to this test were further tested by culturing in LD and ONPG broths. Colonies that showed lysine decarboxylation and an absence of β-D-galactosidase were identified as *Salmonella* (Figure 1).

Identification of nalidixic acid and/or rifampicin resistant strains:

Bacteria identified as *Salmonella* were further investigated to determine their resistance to either nalidixic acid or rifampicin, or both. The bacteria were inoculated onto: 1) SBA plates containing 0.04% nalidixic acid; 2) SBA plates containing 0.02% rifampicin, and; 3) SBA plates containing 0.04% nalidixic acid and 0.02% rifampicin. These plates were incubated at 37°C for 24 to 48 h. Colonies growing on 1) but not growing on 2) and 3) were identified as resistant to nalidixic acid, those growing on 2) but not on 1) and 3) as resistant to rifampicin and those growing on 1), 2) and 3) as resistant to both nalidixic acid and rifampicin.

Polyvalent O antigen slide agglutination test for *salmonella*:

Bacterial colonies were mixed with a drop of sterile PBS to make two heavy suspensions per slide. One of the suspensions was left for one minute to confirm the absence of auto-agglutination, then a drop of PBS was added to the suspension as a control. A drop of *Salmonella* polyvalent O agglutinating serum was mixed with the other bacterial suspension. The slide was then rocked in a rolling motion for one minute. Suspensions in which agglutination occurred within one minute were considered positive.

MIC and MBC of antimicrobial agents:

MIC was defined as the lowest concentration of antimicrobial agents which inhibits visible bacterial growth, and MBC is the lowest concentration of the agents which kills a defined proportion (usually 99.9%) of the bacterial population after incubation for a set time (Collins *et al.*, *Microbiological Methods* pp86, 114-115, 178-205, Butterworth-Heineman, Oxford, England, 1995). The broth dilution method was used for determining MIC. The method for determining MBC was an extension of the broth dilution method.

Determination of bacterial growth rate:

Bioscreen analyser system:

All microorganisms increase the turbidity (optical density (OD)) of the liquid growth medium in which they are being cultured, and for any given type of microorganism, the relationship between the turbidity and the microorganism concentration in a liquid medium is approximately linear over a certain range of values (Metssalu, *A new user's manual*, pp1-27, Labsystems Corporation, Helsinki, Finland, 1995). Bioscreen is a fully automated, turbidmetric instrument to observe *in vitro* growth of microorganisms kinetically or at an end-point. To determine kinetic growth of microorganisms, the turbidity of samples is periodically

monitored at the selected temperatures (room temperature, 37 and 40°C) for the desired period by a vertical light photometry system. In this system, a vertical light beam passes through the bottom of the cuvette, the sample, and the cuvette cover to the photometric reader. A turbidity curve (growth curve) is computer generated based on the results of turbidimetry.

Determination of growth rate:

Unless otherwise stated, the following procedure was used in the project. Bacteria were suspended in a liquid medium to a concentration of approximately 10^8 cfu/ml. In order to prevent an extended lag phase, suspensions were pre-incubated aerobically at 37°C for 3 h (for wild strain FD436) and 5 h (for metabolic-drift mutants). The same type of liquid medium (heated to 37°C) was then added to obtain a concentration of approximately 10^5 cfu/ml. Two hundred microlitres of well-mixed suspension were aseptically transferred into a sterile cuvette. The cuvette was then immediately placed in Bioscreen and the turbidity of the suspension measured at the required temperature over time. A light wavelength filter for a wide band area (420-580 nm) was used because results obtained using this filter are not affected by changes in the colour of the growth media but indicate only the development of turbidity (Metssalu, 1995, *Supra*). The growth rate was taken to be the median value of three simultaneous measurements for each strain.

Replica plate method:

A disk-shaped plate with a stand which fits inside a petri dish was used to aseptically replicate large numbers of colonies growing on one medium to fresh solid media. Before replication, the surface of the disk was tightly covered with a piece of sterile cloth. The covered surface was then applied evenly and precisely to the agar surface containing between 30 and 300 colonies. The disk was then applied evenly to the new media, accurately reproducing colony distribution on the original plate (Carlton and Brown, *Gene Mutation* in *Manual of Methods for General Bacteriology*, pp232-234, Eds Gerhardt *et al.*, American Society for Microbiology, Washington DC, USA, 1981).

Challenge bacteria and vaccine preparation:

Fresh cultures were suspended in PBS to give an approximate concentration of 10^7 cfu/ml. One millilitre of suspension was flooded onto SBA plates, reduced by anaerobic incubation at 37°C overnight, and the plates then anaerobically incubated at 37°C for 4 h (for wild strain FD436) and 6 h (for metabolic-drift mutants). Bacteria growing on each plate were harvested in PBS by gentle washing using a pipette. Bacterial preparations for challenge infection and vaccination were made from these suspensions by adding warm (37°C) PBS to give the desired bacterial concentrations.

Fifty percent infectious dose (ID_{50}):

The ID_{50} is a measurement of microorganism virulence that determines the dose required to infect 50% of the animals in the target group. Groups of forty mice were used to estimate the ID_{50} and to provide a standard deviation from the mean using the Reed-Muench method (Reed and Muench, *The American Journal of Hygiene 27*:493-497, 1937).

Euthanasia of mice:

Five parameters, activity, appetite, dehydration, respiratory rate and faecal consistency, were monitored to assess the health of mice. Mice showing clinical signs in three or more of the five parameters were classed as suffering from serious illness and immediately euthanased using intraperitoneal injection with Nembutal at a dose of 100 µl/mouse.

Statistical methods:

GraphPad Instat version 3.05 (GraphPad Software, San Diego, USA) was used to analyse data. Results were considered statistically significant when probability was less than 5% ($p < 0.05$). Detailed statistical analysis is described in each chapter.

EXAMPLE 2

Isolation of double metabolic-drift mutants

<u>Experimental procedures for Example 2:</u>

Bacteria:

Fresh cultures of FD436 were prepared as previously described.

MIC and MBC determination:

Media:

Two percent (w/v) nalidixic acid solution, or 1% (w/v) rifampicin solution, was added to TSB to make 2 ml of TSB media containing graded concentrations of nalidixic acid or rifampicin (0.04, 0.02, 0.01, 0.005, 0.0025, 0.00125, 0.000625 and 0.0003125% (w/v)). TSB media containing no antimicrobials were also prepared as controls. TSB medium was used instead of the conventionally recommended media for MIC and MBC determinations as metabolic-drift mutants may be more fastidious than their parent strains and thus may require highly nutritious media, such as TSB.

Methods:

Turbidity ($OD_{420-580}$) measured by Bioscreen and visual observation after 24 h incubation at 37°C were the criteria used for assessing the growth of bacteria. FD436 was mixed with the above TSB media containing the graded concentrations of antimicrobials to make approximately 10^6 cfu/ml bacterial suspensions (the actual concentration was 9.6×10^5 cfu/ml). Inoculated and uninoculated TSB containing no antimicrobials were included as positive and negative controls, respectively. For determining the MIC, 200 µl of each medium was aseptically transferred to a sterile cuvette and bacterial growth was measured at 37°C for 24 h using Bioscreen. In addition, the remainder of the medium was incubated

aerobically at 37°C for 24 h for visual observation of turbidity. The MICs of the antimicrobials were determined based on the definition described previously. For determining the MBC, each medium containing antimicrobial concentrations above the MIC was diluted with TSB to the MIC, and 1 ml of each was pipetted directly onto an SBA plate and distributed with a sterile glass spreader. These plates were incubated at 37°C for 48 h. Colonies growing on the SBA were identified using the *Salmonella* identification procedure. The MBC of each antimicrobial was determined based on the definition described previously.

Isolation of single antimicrobial-resistant strains:

Colonies from a fresh culture of FD436 on SBA plates were mixed with PBS to make a bacterial suspension of approximately 10^{10} cfu/ml (the actual concentration was determined to be 2.1×10^{10} cfu/ml). One millilitre of the suspension was spread onto SBA plates containing 0.04% (w/v) nalidixic acid or 0.02% (w/v) rifampicin (three plates each), and a SBA plate containing no antimicrobials as a control. These plates were incubated aerobically at 37°C for 72 h. Bacteria isolated on the plates were identified using the *Salmonella* identification procedure.

Selection of stable, single antimicrobial-resistant strains

Seventeen strains (N1–N14 and R1–R3) were tested for stability of resistance by subculturing a total of 10 times on SBA plates containing 0.04% (w/v) nalidixic acid and 0.02% (w/v) rifampicin, respectively. Each strain was also inoculated onto SBA plates containing no antimicrobials as a control. Plates were incubated aerobically at 37°C for 48 h before the next subculture.

Selection of single antimicrobial-resistant strains with a reduced growth rate:

The turbidity of strains N1–N14 and R1–R3 growing in TSB media at 37°C was measured every 10 min for 18 h using Bioscreen. The determination of turbidity followed the procedure described previously. The turbidity of strain FD436

growing in TSB media was also measured as a control in the same manner. The growth rate during the logarithmic growth phase of each strain was compared.

Isolation of double antimicrobial-resistant strains:

Colonies from fresh cultures of strains N1, N6 and R3 on SBA plates were mixed with PBS to make bacterial suspensions of approximately 10^{10} cfu/ml (actual concentrations were 1.6×10^{10}, 1.1×10^{10}, 1.6×10^{10} and 0.9×10^{10} cfu/ml, respectively). One millilitre of each suspension containing the nalidixic acid-resistant strains was spread onto 0.04% (w/v) nalidixic acid and 0.01% (w/v) rifampicin SBA plates and 0.04% (w/v) nalidixic acid and 0.02% (w/v) rifampicin SBA plates (three plates each). One millilitre of the suspension containing the rifampicin-resistant strain was spread onto 0.02% (w/v) nalidixic acid and 0.02% (w/v) rifampicin SBA plates and 0.04% (w/v) nalidixic acid and 0.02% (w/v) rifampicin SBA plates (three plates each). The same amount of the suspensions was inoculated onto SBA plates containing no antimicrobials as controls. All plates were incubated aerobically at 37°C for 72 h. Bacteria growing on each plate were identified using the *Salmonella* identification procedure.

Selection of stable, double antimicrobial-resistant strains:

Twenty-seven potential N-R double antimicrobial-resistant mutant colonies were randomly selected from 94 colonies growing on 0.04% (w/v) nalidixic acid and 0.02% (w/v) rifampicin SBA plates on which N1 or N6 had been inoculated. As only 2 potential R-N double antimicrobial-resistant mutant colonies were isolated both were selected for further studies. The selected colonies and ID numbers of the colonies (strains) are indicated in Table 6. Each candidate double antimicrobial-resistant strain (1-29) was subcultured 10 times on SBA containing 0.04% (w/v) nalidixic acid and 0.02% (w/v) rifampicin, and on SBA containing no antimicrobials (control). Each subculture was incubated aerobically at 37°C for 48 h. The strains were then subcultured a further 10 times on SBA containing no antimicrobials (each subculture was incubated at 37°C for 48 h). Following this, an isolated colony of each strain was mixed with 2ml of PBS and the serial ten-fold

dilution method was employed to make bacterial suspensions containing a final concentration of approximately 10^2 cfu/ml. One millilitre of each suspension was spread onto individual SBA plates and incubated aerobically at 37°C for 48 h. After incubation, colonies growing on each SBA plate were transferred onto 0.04% (w/v) nalidixic acid and 0.02% (w/v) rifampicin SBA plates and new SBA plates using the replica plate method. The replicated plates were then incubated aerobically at 37°C for 48 h. Approximately 500 colonies of each strain were transferred using the replica plate method.

Selection of double antimicrobial-resistant strains with a reduced growth rate:

The growth rates of double antimicrobial-resistant strains 1-29 were determined based on the results of turbidimetry measurements by using the same procedures as outlined previously.

Results for Example 2:

MIC and MBC determination for FD436:

MIC and MBC of nalidixic acid:

Through turbidity measurements obtained with Bioscreen and direct observation, the MIC of nalidixic acid for FD436 was determined to be 0.00125% (w/v) (Table 3). The MBC was also determined to be 0.00125% (w/v) as profuse growth (greater than 1.0×10^3 colonies) of *Salmonella* was observed on SBA plates streaked with TSB media originally containing equal to or less than 0.000625% (w/v) of nalidixic acid. Only small numbers of colonies or no colonies at all were found on SBA plates streaked with TSB media originally containing equal to or greater than 0.00125% (w/v) of the antimicrobial (Table 4).

MIC and MBC of rifampicin:

Through testing with Bioscreen and direct observation, the MIC of rifampicin for FD436 was determined to be 0.0025% (w/v) (Table 3). The MBC was also determined to be 0.0025% (w/v) (Table 4).

TABLE 3: TURBIDITY OF TSB MEDIA CONTAINING NALIDIXIC ACID OR RIFAMPICIN AFTER 24-HOUR INCUBATION MEASURED USING BIOSCREEN

Concentration of antimicrobial in TSB (% (w/v))	Nalidixic acid	Rifampicin
0.04	–	–
0.02	–	–
0.01	–	–
0.005	–	–
0.0025	–	–
0.00125	–	+
0.000625	+	+
0.0003125	+	+
Positive control	+	+
Negative control	–	–

+ = increase, – = no change

TABLE 4 : GROWTH OF *Salmonella* ON SBA PLATES AFTER 48-HOUR INCUBATION IN TSB MEDIA CONTAINING NALIDIXIC ACID OR RIFAMPICIN.

Concentration of antimicrobial in TSB (% (w/v))	Nalidixic acid	Rifampicin
0.04	1	N.G
0.02	8	1
0.01	N.G	1
0.005	N.G	5
0.0025	1	6
0.00125	N.G	P.G
0.000625	P.G	P.G

0.0003125	P.G	P.G
Positive control	P.G	P.G
Negative control	N.G	N.G

N.G = no growth, P.G = profuse growth (greater than 1.0×10^3 colonies)

Isolation and stability of single antimicrobial-resistant strains:

In total, 14 colonies were observed on the SBA plates containing 0.04% (w/v) nalidixic acid after 72 h of incubation. After 72 h of incubation, 3 colonies were found on the SBA plates containing 0.02% (w/v) rifampicin. Similar to the growth observed on the 0.04% (w/v) nalidixic acid SBA plates, the colonies on the 0.02% (w/v) rifampicin SBA plates took more time to appear than the colonies on the control plate. Each isolated colony was identified as a strain of *Salmonella*. Strains from SBA plates containing nalidixic acid were designated N1–N14 and strains from SBA plates containing rifampicin were designated R1–R3.

All strains grew well on SBA plates containing the antimicrobials throughout the ten subcultures.

Selection of single antimicrobial-resistant strains with a reduced growth rate:

Nalidixic acid resistant strains N1 and N6 had slower growth rates when compared with wild strain FD436 (Figure 2). Rifampicin resistant strain R3 also had a reduced growth rate when compared with FD436 (Figure 3). Based on these results, N1, N6 and R3 were selected for producing double antimicrobial-resistant strains.

Isolation of double antimicrobial-resistant strains:

For N1 and N6, the total number of potential double antimicrobial-resistant mutant colonies ranged between 38 and greater than 100 for each antimicrobial concentration after 72 h of incubation. For R3, only one colony grew on each plate for each antimicrobial concentration after 72 h of incubation (Table 5). Profuse growth was seen on each control plate. Bacteria growing on the plates were identified as *Salmonella*.

TABLE 5 : NUMBER OF POTENTIAL DOUBLE ANTIMICROBIAL-RESISTANT MUTANT COLONIES (N-R AND R-N) GROWING ON SBA PLATES CONTAINING DIFFERENT CONCENTRATIONS OF NALIDIXIC ACID AND RIFAMPICIN AFTER 72-HOUR INCUBATION.

Inoculated strain	Concentration (w/v) of antimicrobials in media	Number of colonies*
N1	0.04% nalidixic acid & 0.01% rifampicin	> 100
N1	0.04% nalidixic acid & 0.02% rifampicin	56
N1	No antimicrobials (control)	P.G
N6	0.04% nalidixic acid & 0.01% rifampicin	59
N6	0.04% nalidixic acid & 0.02% rifampicin	38
N6	No antimicrobials (control)	P.G
R3	0.02% nalidixic acid & 0.02% rifampicin	1
R3	0.04% nalidixic acid & 0.02% rifampicin	1
R3	No antimicrobials (control)	P.G

P.G = profuse growth, N.G = no growth, * = Numbers indicate the total number of colonies on 3 plates.

Selection of stable, double antimicrobial-resistant strains:

The twenty-nine potential double antimicrobial-resistant strains (Table 6) grew well and maintained their double antimicrobial resistance throughout and following the ten subcultures on SBA plates containing 0.04% (w/v) nalidixic acid and 0.02% (w/v) rifampicin. Therefore, the selected strains have obtained highly stable resistance to both nalidixic acid and rifampicin.

TABLE 6 : CANDIDATE DOUBLE ANTIMICROBIAL-RESISTANT STRAINS SELECTED IN ORDER TO DETERMINE THE STABILITY OF RESISTANCE.

Parent single antimicrobial-resistant strain	Concentration (w/v) of antimicrobials in media	Order of obtained antimicrobial-resistance	Number of selected colonies	ID number
N1	0.04% nalidixic acid & 0.02% rifampicin	N-R	12	1-12
N6	0.04% nalidixic acid & 0.02% rifampicin	N-R	15	13-27
R3	0.02% nalidixic acid & 0.02% rifampicin	R-N	1	28
R3	0.04% nalidixic acid & 0.02% rifampicin	R-N	1	29

Selection of double antimicrobial-resistant strains with a reduced growth rate:

Compared with wild strain FD436, the growth rates of both the N-R and the R-N double antimicrobial-resistant strains were reduced to different levels (Figures 4 and 5).

EXAMPLE 3

Characterization of double antimicrobial-resistant strains with reduced growth rate and selection of vaccine candidates

Experimental procedures for Example 3:

Bacteria:

FD436 and the 8 selected double antimicrobial-resistant strains N-RM4, 8, 9, 15, 20, 25, and 27 and R-NM29 were prepared following the procedure described previously.

Genotyping characterization:

Genotypic characterisation of FD436 and the double antimicrobial-resistant strains prepared as above was performed. To investigate the stability of the identified mutations, characterisation of the strains was repeated following subculturing 15 times on SBA (each subculture was incubated at 37°C for 48 h).

PCR amplification of *S.dublin gyrA* genes:

A portion of the *gyrA* gene (expected approximate size 347 base pairs (bp) (Griggs *et al., Antimicrobial Agents and Chemotherapy 40*:1009-1013, 1996) encompassing the QRDR region was amplified by PCR for FD436 and the 8 mutant strains using 20-mer oligonucleotide primers, SALGYRA-F and SALGYRA-R (synthesized by Genset Pacific Pty., Ltd., Lismore, Australia). The primer sequences were as follows - SALGYRA-F = 5'-TGTCCGAGATGGCCTGAAGC-3' (SEQ ID NO:1) and SALGYRA-R = 5'-TACCGTCATAGTTATCCACG-3' (SEQ ID NO:2). The PCR amplification reactions (total 50 µl) contained 6.4 picomole (pmol) of each primer, 200 µM of each dinucleotide triphosphate (dNTP), 1× Expand High Fidelity buffer with 2 mM magnesium chloride (Expand High Fidelity PCR System, Roche Diagnostics Australia Pty., Ltd., Australia), 1 unit of Expand High Fidelity PCR enzyme mix (Expand High Fidelity PCR System) and sterile distilled water. For ease and rapidity, all PCR assays were performed directly using single colonies grown on SBA plates as the DNA target. A small amount of a colony was taken by touching it lightly with a small pipette tip, and this sample was then resuspended in the PCR amplification reaction. All amplification reactions were performed in a Gene Amp PCR System 2400 thermocycler (PerkinElmer, USA). After an initial denaturation at 94°C for 3 min, the cycling protocol for 30 cycles was as follows: denaturation at 94°C for 30 seconds (sec), annealing at 55°C for 30 sec and extension at 68°C for 3 min. A final extension was conducted at 72°C for 3 min.

PCR amplification of *S.dublin rpoB* genes:

A portion of the *rpoB* gene (expected approximate size 708 bp (McClelland *et al., Nature 413*:852-856, 2001)) was amplified by PCR for FD436 and the mutant strains. This portion encompassed three distinct clusters (I, II and III) that Jin and Gross (*Journal of Molecular Biology 202*:45-58, 1988) suggest are related to rifampicin resistance. Materials and methods for this amplification were identical to those described previously for PCR amplification of the *gyrA* gene with the

exception of the type of primers used. In this experiment, 20-mer oligonucleotide primers, SALRPOF3 and RPOBREV (Genset Pacific Pty., Ltd. and Gibco BRL, respectively) were used. The primer sequences were as follows - SALRPOF3 = 5'-TCGGCAACCGTCGTATCCGT-3' (SEQ ID NO:3) and RPOBREV = 5'-TCGCACCCATCAACGCACGG-3' (SEQ ID NO:4).

Agarose gel electrophoresis:

Amplified DNA fragments were separated by agarose gel electrophoresis in 2% and 1.5% agarose gels containing 1% ethidium bromide (Bio-Rad Laboratories Ltd., Sydney, Australia). The gels were prepared by dissolving the desired amounts of DNA Grade Agarose in 1× TAE buffer (40 mM Tris base) and 1 mM EDTA titrated to pH 7.9 with acetic acid) by heating in a microwave oven. The dissolved agarose was then cooled to approximately 50°C and poured onto a flatbed horizontal gel apparatus (Bio-Rad Laboratories Ltd.). Before use, the agarose was kept at room temperature for 30 min to allow polymerisation.

Following the above amplifications, 5 µl of each PCR product was mixed with 1 µl of tracking dye solution (50% glycerol, 0.1% bromophenol blue and 0.1% xylene cyanol FF (Australian Chemical Reagents, Australia)), loaded into a well and electrophoresed in the agarose gels (2% for *gyrA* products and 1.5% for *rpoB* products) at 60 mA for 1 h. A 1 kbp DNA ladder (MBI Fermentas, USA) was used as a standard. Amplified DNA fragments were visualised by UV transillumination.

DNA sequencing and analysis:

Prior to DNA sequencing, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) following the procedure described in the manufacturer's manual. The oligonucleotide primers, SALGYRA-F and SALGYRA-R, and SALRPOF3 and RPOBREV, were used as primers for direct sequencing of the amplified *gyrA* and *rpoB* gene fragments, respectively. Each sequencing reaction mixture (total 12 µl) contained 4 µl of BigDye Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, USA), 3.2 pmol

of primer, 1 µl of purified PCR product (DNA product) and sterile distilled water. Sequencing reactions were performed using the following cycling conditions: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 4 min. Products of the sequencing reactions were purified using the following procedure. Each sequencing reaction mixture (12 µl) was mixed with 50 µl of ice-cold 100% ethanol and 2 µl 3M sodium acetate (Sigma), and kept at –20°C for 30 min. The mixtures were then centrifuging performed (13,000 rpm at 4°C) for 30 min and the supernatant was gently discarded to obtain a pellet containing the sequencing reaction product. Five hundred microliters of 70% ethanol was added and centrifuged again (13,000 rpm, 4°C, 30 min). Supernatant was carefully discarded and the pellet was air-dried. The reaction product was analysed using a 373A DNA Sequencing System at the Australian Genome Research Facility (AGRF), Brisbane, Qld.

Phenotypic characterizaiton:

Colony:

Colonies on SBA plates seeded with each of the 9 strains and incubated aerobically for 24 h at 37°C were examined under 10× magnification.

Motility:

The conventional hanging drop method (Frost and Spradbrow, *Veterinary Microbiology I, II, II Practical Manual and Laboratory Notes*, p8, The University of Queensland, Brisbane, Australia) and the Craigie tube method (Collins *et al.*, *Supra*) were used to examine the motility of FD436 and the selected mutants.

For the Craigie tube method, 12 ml of semi-solid medium was distributed into one oz screw-capped bottles containing an open-ended glass tube (the Craigie tube) to make a Craigie tube set. These sets were then autoclaved at 121°C for 15 min. One individual colony of each strain was inoculated approximately 10 mm below the surface of the semi-solid medium within the Craigie tube using a sterile straight

wire. The media were then incubated at 37°C for 36 h. Samples of each strain were taken every 3 h from the surface of the media outside the Craigie tube and cultured on XLD plates. To avoid false positive results produced by bacterial contamination, organisms growing on the XLD media were identified as being *Salmonella* using the procedure described previously. Each result was the mean of two separate determinations per strain.

Capsulation:

Duguid's method (Doetsch, Determinative Methods of Light Microscopy In *Manual of Methods for General Bacteriology*, p29, Eds Gerhardt, *et al.*, American Society for Microbiology, Washington DC, USA, 1981) was used to determine capsulation. Briefly, a large loopful of India ink was mixed with cells from each strain on a clean slide and then pressed down with a glass cover slip. Excess mixture was absorbed by blotting paper. Capsulation was examined with high-dry (×400) and oil immersion lens systems.

Lipopolysaccharides (LPS)

Lipopolysaccharides extracts were resolved by SDS-PAGE and silver stained to detect and evaluate heterogeneity of LPS structure between the strains.

Extraction of LPS:

Proteinase K digestion (Hitchcock and Brown, *Journal of Bacteriology 154*:269-277, 1983) followed by phenol-water extraction (Marolda *et al.*, *Journal of Bacteriology 172*:3590-3599, 1990) was employed to extract LPS. Colonies from fresh cultures of each strain were suspended in PBS (pH 7.2) and the concentration adjusted to approximately 10^9 cfu/ml. To sediment bacteria, 1.5 ml of each suspension was centrifuged at 10,000 × g for 3 min. Supernatant fluid was carefully discarded and the pellet was resuspended in 50 μl of solubilisation buffer (Table 7). This suspension was heated at 100°C for 10 min. Twenty-five micrograms of

proteinase K (Roche Diagnostics GmbH, Germany) was then added to the suspension and incubated at 60°C for 1 h. The suspension was extracted with double the volume of TE Saturated Phenol/Chloroform (Progen Industries Ltd.) by stirring vigorously at 65°C for 15 min. The suspension was then chilled on ice and centrifuged at 8,500 × g for 10 min. The aqueous phase was transferred to a new microcentrifuge tube, and LPS was precipitated twice with 10 volumes of 95% ethanol at –20°C for 6 h. The precipitate was suspended in 50 µl of DDW and stored at –20°C until use.

SDS-PAGE of LPS extracts:

SDS-PAGE was performed for the selected mutants and the wild strain using the bi-layer stacking gel method (Inzana and Apicella, *Electrophoresis 20*:462-465, 1999) with some modifications. Gels were cast in 18 cm × 16 cm glass plates separated with 0.75 mm spacers. The separating gel (Table 6) was poured to 4 cm from the top of the plates. After the separating gel polymerised, stacking gel A (Table 6) was poured onto the separating gel to a level at which the well comb just touched stacking gel A. After polymerisation, the well comb (0.75 mm) was placed in the gel sandwich and then overlaid with stacking gel B (Table 6) until all the teeth were covered by the gel. The well comb was carefully removed after polymerisation of the gels and the wells were filled with reservoir buffer (0.025M Tris base/0.19M glycine, pH 8.3) containing 0.1% sodium dodecyl sulfate (SDS).

Prior to running the gel, extracted LPS of each sample in 50 µl of DDW was mixed with the same amount of solubilisation buffer and placed in boiling water for 5 min. Fifty microliters of each mixture was loaded into the well. Electrophoresis was performed at 4°C using a circulating cooling bath in a Protean II vertical electrophoresis unit (Bio-Rad Laboratories Ltd.). The samples were electrophoresed at a constant current of 10 mA/gel through both stacking gels, then 15 mA/gel through the separating gel. Two gels were prepared for the samples and electrophoresed at the same time under the same conditions. One of the gels was stained with ammoniacal silver for detecting LPS. To confirm complete digestion

of protein in the samples by Proteinase K, the other gel was stained with Coomassie Brilliant Blue stain (10% (v/v) methanol, 10% (v/v) acetic acid and 0.1% (w/v) Coomassie Blue R-250 (Sigma).

Silver staining:

After electrophoresis, the gel was fixed and stained with ammoniacal silver following the method described by Tsai and Frasch (*Analystical biochemistry 119*:115-110, 1982) with some modification. The gel was fixed overnight with 200 ml of 40% (v/v) ethanol and 10% (v/v) acetic acid in DDW. The gel was then oxidised for 5 min in 200 ml of the above fixative containing 0.7% (w/v) periodic acid (Sigma), and washed with DDW three times for 15 min each. The gel was stained for 10 min with a freshly prepared stain consisting of 0.25 ml of 30% sodium hydroxide (Ajax), 1.4 ml of 14.8M ammonium hydroxide (Sigma), 4 ml of 20% silver nitrate (Ajax) and 97 ml of DDW. After staining, the gel was washed with DDW three times for 10 min each and then developed with 0.005% (w/v) citric acid (Ajax) and 0.019% (v/v) formaldehyde (Asia Pacific Specialty Chemicals Ltd., Australia) in DDW. When bands were visible, the development was stopped by removing the gel and soaking in the fixative.

TABLE 7 : COMPOSITION OF SOLUBILISATION BUFFER AND SEPARATING AND STACKING GELS.

Solution	Component	Volume (ml)
Solubilisation buffer	Stock buffer (0.06M Tris-HCl, pH6.8, 1 mM EDTA, 2% SDS)	0.59
	Glycerol	0.40
	2-mercapto-ethanol	0.08
	Saturated bromophenol blue	0.04
Separating gel	30% Acrylamide stock (29.2% acrylamide and 0.8% bis-acrylamide (Bio-Rad Laboratories Ltd.))	9.30
	9M Urea (BDH)	6.40
	1.88M Tris/HCl, pH 8.8	4.00
	10% SDS	0.20
	Degas 10 min, then add	
	10% Ammonium persulfate (Sigma)	0.15
	N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma)	0.01
Stacking gel A	30% Acrylamide stock (29.2% acrylamide and 0.8% bis-acrylamide)	1.00
	1.25M Tris/HCl, pH 6.8	1.00
	10% SDS	0.10
	DDW	7.80
	Degas 10 min, then add	
	10% Ammonium persulfate	0.10
	TEMED	0.01
Stacking gel B	30% Acrylamide stock (29.2% acrylamide and 0.8% bis-acrylamide)	1.00
	0.25M Tris/1.92M glycine, pH 8.3	1.00
	10% SDS	0.10
	DDW	7.80
	Degas 10 min, then add	
	10% Ammonium persulfate	0.10
	TEMED	0.01

5 Temperature sensitivity:

Each of the 9 strains was inoculated onto two SBA plates. One of the two plates was acrobically incubated at 20°C and the other at 42°C. The growth of each strain was examined at 48 h after inoculation.

Hydrogen sulphide production:

Bacteria from a single colony of each strain on an SBA plate were inoculated onto an XLD plate and to TSI agar using conventional methods. They were then incubated at 37°C for 48 hours. Strains that produced black pigment in TSI agar media and in the centre of isolated colonies on XLD media were identified as hydrogen sulphide producers.

Lysine decarboxylase (LD) test:

Bacteria of each strain on an SBA plate were inoculated into LD broth medium and incubated at 37°C for 48 hours. Strains that showed a change in colour of the media to dark purple were identified as producers of LD.

O-nitrophenyl-β-d-galactopyranoside (ONPG) test:

Bacteria of each strain on an SBA plate were inoculated into an ONPG broth medium and incubated at 37°C for 48 hours. Strains that grew in the media without colour change were determined to be lacking galactosidase.

Fermentation of carbohydrates:

Twenty different kinds of carbohydrate fermentation broth media were prepared following the procedure described previously. Carbohydrates used in this experiment were as follows:

Adonitol [Sigma, A-5502]
D-(-)-Arabinose minimum 99% [Sigma, A-3131]
L-(+)-Arabinose minimum 99% [Sigma, A-3256]
D-(+)-Cellobiose minimum 98% [Sigma, C-7252]
Dulcitol [Sigma, D-0256]
D-(+)-Glucose 99.5% [Sigma, G-7528] (GC)
Inositol (Meso) [BDH, 38044] (AR)
Lactose [BDH, 10139] (AR)

Maltose [BDH, 29131] (Laboratory reagent (LR))
Mannitol [Ajax, D-3247] (LR)
D-(+)-Mannose [BDH, 01566]
Melibiose [Sigma, M-5500]
D-(+)-Raffinose [Sigma, R-0250]
α-L-Rhamnose [Sigma, R-3875]
Salicin [BDH, 38060]
D-Sorbitol minimum 98% [Sigma, S-1876]
Sucrose 99+% [Sigma, S-0389] (AR)
D-(+)-Trehalose [Sigma, T-5251]
D-(+)-Xylose [Sigma, X-1500]
L-(-)-Xylose [Sigma, X-1625]

To investigate gas production in a broth medium containing D-(+)-glucose, a small vial (5mm × 25 mm) was placed in an inverted position in a 1/4 oz bottle in which 2 ml of the medium had been distributed. After autoclaving at 121°C for 15 min, the vial was completely filled with the medium (Smibert and Krieg, General Characterization In *Manual of Methods for General Bacteriology*, p415, Eds Gerhardt *et al.*, American Society for Microbiology, Washington DC, USA, 1981).

One individual colony of each of the 9 strains was inoculated into each broth medium containing a different carbohydrate. The media were then incubated at 37°C for 10 days. The media were observed for red colour change and gas production every 24 h over the incubation period.

Proteins:

Protein profiles of the strains were analysed using SDS-PAGE.

Extraction of whole cell proteins:

A fresh culture of each strain on SBA was harvested in 2 ml of 0.1M Tris/HCl (pH 6.8) containing 15% glycerol, 2mM phenyl methyl-sulfonyl fluoride (PMSF)

(BDH) and 2% SDS to obtain an approximate concentration of 10^{10} cfu/ml. The suspensions were boiled in a water bath for 5 min and whole cell protein extracts collected and stored at –20°C until use.

SDS-PAGE of whole cell protein extracts:

A 12% acrylamide separating gel and a 4% acrylamide stacking gel (Table 7) were used for the separation of bacterial proteins. Prior to running the gel, each sample was mixed with 2× SDS sample buffer (Table 7) at a ratio of 2:1. The mixture was then placed in boiling water for 5 min. Fifteen microliters of each mixture was loaded into the well. Electrophoresis was performed in a running buffer (0.025M Tris base/0.19M glycine (pH 8.3) containing 0.1% SDS) using a circulating cooling bath (at 10°C) in a Mini Protean vertical electrophoresis unit (Bio-Rad Laboratories Ltd.) at 30 mA for 1 h. Following electrophoresis, the separating gel was stained with 0.1% Coomassie Brilliant Blue for an hour.

TABLE 8 : COMPOSITION OF SEPARATING AND STACKING GELS AND SAMPLE BUFFER.

Solution	Component	Volume (ml)
Separating gel	30% Acrylamide stock (29.2% acrylamide and 0.8% bis-acrylamide)	5.00
	1.5M Tris/HCl, pH 8.8	3.13
	10% SDS	0.13
	DDW	4.18
	Degas 10 min, then add	
	10% Ammonium persulfate	0.06
	TEMED	0.006
Stacking gel	30% Acrylamide stock (29.2% acrylamide and 0.8% bis-acrylamide)	0.65
	0.5M Tris/HCl, pH 6.8	1.25
	10% SDS	0.05
	DDW	3.02
	Degas 10 min, then add	
	10% Ammonium persulfate	0.03
	TEMED	0.005
2× SDS sample buffer	0.5M Tris/HCl, pH 6.8	1.00
	Glycerol	0.80
	10% SDS	1.60
	2-mercapto-ethanol	0.40
	DDW	0.40
	Saturated bromophenol blue	0.20

Antimicrobial sensitivity:

Antimicrobial agents:

Commercial antimicrobial disks (Oxoid) were used for susceptibility tests. Antimicrobial agents and the amount contained in each disk are shown in Table 9.

TABLE 9 : ANTIMICROBIAL AGENTS AND DISK CONTENTS USED IN DISK SUSCEPTIBILITY TESTS.

Group	Agent	Content (μg)
β-lactams penicillin	Amoxicillin	10
β-lactams/β-lactamase inhibitor combination	Amoxicillin/Clavulanic acid	20/10
	Ticarcillin/Clavulanic acid	75/10
Cephalosporin (1st)	Cephalothin	30
Cephalosporin (3rd)	Ceftazidime	30
Aminoglycoside	Gentamicin	10
Tetracycline	Tetracycline	30
Fluoroquinolones	Enrofloxacin	5
	Ciprofloxacin	5
Others	Sulphamethoxazole/Trimethoprim	1.25/23.75

1st = first generation; 3rd = third generation.

Antimicrobial disk susceptibility tests:

The procedure employed in the tests was in accordance with the method proposed by The National Committee on Clinical Laboratory Standards (NCCLS) (Ferraro *et al.*, Performance standards for antimicrobial susceptibility testing, *NCCCLS 18*:1-35, 1998; Watts *et al.*, Performance standards for antimicrobial disk and dilution susceptibility test for bacteria isolated from animals, NCCLS *17*:1-63, 1994). Briefly, MHA medium was dispensed into plastic culture plates with 9 cm internal diameters and flat bottoms to yield a uniform depth of 4 mm. These plates were kept at room temperature for 1 h and then dried in an incubator at 35°C for 30 min. Four isolated colonies from a fresh culture of each strain were suspended in 4ml of TSB and incubated at 37°C for 3 h (for wild strain FD436) and 5 h (for mutant strains). PBS heated to 37°C was then added to the suspensions to give approximately 10^8 cfu/ml (comparable with the 0.5 McFarland turbidity standard). Each mixture was spread onto an MHA plate by streaking 3 times using a sterile cotton swab, and antimicrobial disks were applied to the plates with a dispenser (Oxoid). The inoculated plates were incubated at 37°C for 18 h in an inverted position. Zone diameters for individual antimicrobials were translated in terms of susceptible, intermediate or resistant categories by referring to the zone diameter

interpretative standards proposed by NCCLS (Ferraro *et al.*, 1998, *Supra*; Watts *et al.*, 1994, *Supra*).

Results for Example 3:

Genotypic characterization:

PCR amplification and nucleotide sequence of *S. dublin gyrA* genes:

Based on the results of *gyrA* gene sequencing, the size of the amplified products of FD436 and all tested mutant strains was 347 bp (Figure 6 and Table 10). A nucleotide substitution of A → G was identified in all tested N-RM strains. These substitutions resulted in an amino acid substitution of Asp-87 → Gly. R-NM29 had a substitution of G → T, resulting in a Gly-81 → cysteine (Cys) substitution. Sequencing repeated following 15 times subculture produced identical results.

PCR amplification and nucleotide sequence of S. *dublin rpoB* genes:

Based on the results of *rpoB* gene sequencing, products between the size of 696 and 714 bp were amplified from FD436 and the mutant strains (Figure 7 and Table 11). N-RM4 had two point mutations, nucleotide substitutions of C → T and C → G, which resulted in amino acid substitutions of proline (Pro)-564 → Leu and Gln-649 → glutamic acid (Glu), respectively. N-RM15 showed a single point mutation, a nucleotide substitution of A → T, which caused an amino acid substitution of Ile-572 → Phe. N-RM20 also had a nucleotide substitution of C → A that resulted in an amino acid substitution of Arg-529 → Ser. Both N-RM25 and R-NM29 had a nucleotide substitution of A → G at the same position that resulted in an amino acid substitution of Asp-516 → Gly. A deletion mutation of 12 nucleotides, TCTCAGTTTATG, which resulted in a deletion of 4 amino acids, Ser-512, Gln-513, Phe-514 and methionine (Met)-515, was observed in both N-RM8 and N-RM9. N-RM27 showed an insertion mutation of 6 nucleotides, GACCAG, which

caused an insertion of 2 amino acids, Asp and Gln, between Met-515 and Asp-516. Pre- and post-subculture sequence results for each respective strain were identical.

TABLE 10 : SEQUENCE ANALYSIS OF THE *gyrA* GENE OF *S. dublin* DOUBLE ANTIMICROBIAL-RESISTANT STRAINS.

Strain	Nucleotide change	Amino acid change
N-RM strains[1]	GAC → GGC	Asp-87 → Gly
R-NM29	GGC → TGC	Gly-81 → Cys

[1]: N-RM4, 8, 9, 15, 20 25 and 27; Asp = aspartic acid, Gly = glycine, Cys = cysteine.

TABLE 11 : SEQUENCE ANALYSIS OF THE *rpoB* GENE OF *S. dublin* DOUBLE ANTIMICROBIAL-RESISTANT STRAINS.

Strain	Nucleotide change	Amino acid change
N-RM4	CCG → CTG CAG → GAG	Pro-564 → Leu Gln-649 → Glu
N-RM8	Deletion of TCTCAGTTTATG	Deletion of Ser-512, Gln-513, Phe-514, Met-515.
N-RM9	Deletion of TCTCAGTTTATG	Deletion of Ser-512, Gln-513, Phe-514, Met-515.
N-RM15	ATC → TTC	Ile-572 → Phe
N-RM20	CGT → AGT	Arg-529 → Ser
N-RM25	GAC → GGC	Asp-516 → Gly
N-RM27	Insertion of GACCAG	Insertion of Asp and Gln between Met-515 and Asp-516
R-NM29	GAC → GGC	Asp-516 → Gly

Arg = arginine, Asp = aspartic acid, Gln = glutamine, Glu = glutamic acid, Gly = glycine, Ile = isoleucine, Leu = leucine, Met = methionine, Phe = phenylalanine, Pro = proline, Ser = serine.

Phenotypic characterization:

Colony:

FD436 produced white-grey, opaque, circular, convex colonies with entire margins. The surface of the colonies was smooth and not haemolytic. The mutants, on the other hand, produced smaller colonies after 24-hour incubation than did FD436. Colonies of N-RM 20, 25 and 27 were particularly small. N-RM25 produced grey, translucent, slightly raised colonies that were easily distinguishable from colonies produced by FD436 and other tested mutants (Figure 8). Details are shown in Table 12.

Motility:

The results of the hanging drop method confirm that FD436 and all tested mutants were motile. Using the Craigie tube method, the time until the mutants were first isolated from the surface of the media outside the Craigie tubes was found to range from 12-33 h, longer than the time to first isolation for FD436 (9 h). No bacterial contamination of semi-solid media was evident during the Craigie tube test. Based on these results, motility was divided into three categories (time between inoculation and the first isolation: <12 h = high motility, 12 h - 24 h = medium motility, >24 h = low motility). The results of this experiment are indicated in Table 13.

Capsulation:

The wild strain was not capsulated, nor did any tested mutant strain possess a capsule.

LPS:

Regular ladder-like bands approximately 12 - 17 kDa in size (based on the migration of the protein marker) together with leading bands of approximately 8

kDa in size were observed in the LPS profiles of all strains tested except N-RM20. N-RM20 did not yield a regular banding pattern and the leading band of this strain migrated beyond 8 kDa (Figure 9). No bands were observed on the gel stained with Coomassie Brilliant Blue except in the lane loaded with a protein marker.

Growth:

Temperature sensitivity:

All tested strains grew on SBA media at both 20°C and 42°C.

TABLE 12 : COLONY MORPHOLOGY OF WILD STRAIN FD436 AND MUTANTS N-RM4, 8, 9, 15, 20, 25, 27 AND R-NM29 POST 24-HOUR INCUBATION AT 37°C ON SBA.

	Size[1]	Form	Elevation	Margin	Colour & opacity	Surface
FD436	L	Circular	Convex	Entire	Grey & opaque	Glossy
N-RM4	M	Circular	Convex	Entire	Grey & opaque	Glossy
N-RM8	S	Circular	Convex	Entire	Grey & opaque	Glossy
N-RM9	S	Circular	Convex	Entire	Grey & opaque	Glossy
N-RM15	S	Circular	Convex	Entire	Grey & opaque	Glossy
N-RM20	T	Circular	Convex	Entire	Grey & opaque	Glossy
N-RM25	T	Circular	Raised	Entire	Grey & translucent	Matt
N-RM27	T	Circular	Convex	Entire	Grey & opaque	Glossy
R-NM29	M	Circular	Convex	Entire	Grey & opaque	Glossy

[1]: Average diameter of 5 isolated colonies of each strain. L = > 2 mm; M = 1 – 2 mm; S = 0.5 – 1 mm; T = < 0.5 mm.

TABLE 13 : CHRONOLOGICAL OBSERVATION OF THE MOTILITY OF WILD STRAIN FD436 AND MUTANTS N-RM4, 8, 9, 15, 20, 25, 27 AND R-NM29 USING THE CRAIGIE TUBE METHOD.

First isolation of *Salmonella* post-inoculation (h)	Strain	Motility
9	FD436	High
12	N-RM8, 9, 15	High
15	R-NM29	Medium
18	N-RM 4	Medium
27	N-RM20	Low
30	N-RM25	Low
33	N-RM27	Low

Time periods between inoculation and the first isolation of *Salmonella* strains from the surface of media outside the Craigie tubes: ≤12 h = high motility, 12 h - 24 h = medium, ≥24 h = low.

Biochemical reactions:

Hydrogen sulphide production:

All tested strains produced black pigment both on XLD plates and in TSI agar slopes. Therefore, these strains were identified as hydrogen sulphide producers. However, although N-RM25 was similar to other tested strains in that it produced black pigment in a TSI agar slope within 24 h post-inoculation, production of hydrogen sulphide was markedly slower (taking almost 72 h) on XLD medium.

LD test:

All tested LD broth media turned yellow within a few hours post-inoculation and then turned dark purple. Therefore, the strains were identified as LD producers.

ONPG test:

At 48 hours following inoculation, no colour change in the ONPG broth media was observed, confirming that the tested strains lack galactosidase.

Fermentation of carbohydrates:

Variable results were obtained in D-(-)-arabinose and L-(+)-arabinose fermentation by FD436 and the mutants (Table 14), whereas fermentation of the other carbohydrates was consistent between all strains (Table 15). All tested N-RM strains were negative for gas production in the D-(+)-glucose fermentation test, whereas FD436 and R-NM29 were positive (Table 14).

TABLE 14 : FERMENTATION OF D-(-)-ARABINOSE AND L-(+)-ARABINOSE AND GAS PRODUCTION FROM D-(+)-GLUCOSE FERMENTATION OF WILD STRAIN FD436 AND MUTANTS N-RM4, 8, 9, 15, 20, 25, 27 AND R-NM29.

TEST	FD 436	4	8	9	15	20	25	27	29
D-(-)-arabinose fermentation	+	+	−	−	−	−	−	−	−
L-(+)-arabinose fermentation	+	−	−	−	+	−	−	−	+
Gas production	+	−	−	−	−	−	−	−	+

+ = positive for fermentation or gas production, − = negative for fermentation or gas production.

TABLE 15 : CARBOHYDRATE FERMENTATION TESTS WITH WHICH THE SAME RESULTS WERE OBTAINED FOR FD436, N-RM4, 8, 9, 15, 20, 25, 27 AND R-NM29.

Carbohydrate	Fermentation	Carbohydrate	Fermentation
Adonitol	−	Melibiose	+
D-(+)-cellobiose	−	D-(+)-raffinose	−
Dulcitol	+	α-L-rhamnose	+
D-(+)-glucose	+	Salicin	−
Inositol	−	D-sorbitol	+
Lactose	−	Sucrose	−
Maltose	+	D-(+)-trehalose	+
Mannitol	+	D-(+)-xylose	+
D-(+)-mannose	+	L-(-)-xylose	−

+ = positive for fermentation, − = negative for fermentation.

Metabolism:

Proteins:

The estimated molecular weights of the major protein bands identified in SDS-PAGE for each strain ranged from approximately 18 kDa to 98 kDa (Figure 10). There were 12 main protein bands with molecular weights of approximately 18, 20, 30, 35, 38, 41, 47, 53, 60, 70, 80 and 98 kDa that appeared to be shared among all tested strains. However, the 53 kDa band of R-NM29 was more intensely stained than that of wild strain FD436 and N-R mutants. In addition, although a band of approximately 24 kDa was observed in the protein profiles of the wild strain and the N-R mutants, no such band was detected in the protein profile of R-NM29.

Antimicrobial sensitivity:

FD436 and the mutants, except N-RM20, were susceptible to all antimicrobial agents used in the experiment. N-RM20 showed resistance to enrofloxacin.

EXAMPLE 4

Bile sensitivity as a selection marker for vaccine strains

<u>Experimental procedures for Example 4:</u>

Bacteria:

Selected mutants N-RM4, N-RM25 and R-NM29 were prepared for the experiments following the procedure described previously. FD436 was also prepared as a control in the same manner.

MIC AND MBC determination:

Media:

Two millilitre aliquots of TSB media containing graded concentrations of BS No. 3 were prepared for the experiment. The concentrations of BS No. 3 used were 0.075,

0.15, 0.3, 0.6, 1.2, 2.4, 4.8, 9.6, 14.4, 19.2 and 24% (w/v). TSB media containing no BS No. 3 were also prepared as positive controls.

Methods:

Approximately 10^5 cfu/ml of each strain was suspended in TSB media containing different concentrations of BS No. 3 (the actual numbers of cells as determined by viable bacteria counts were FD436: 2.5×10^5, N-RM4: 1.7×10^5, N-RM25: 1.5×10^5 and R-NM29: 2.0×10^5 cfu/ml). Uninoculated media served as a negative control. For determining MICs, 200 µl of each medium was aseptically transferred to a sterile cuvette and the turbidity ($OD_{420-580}$) was measured at 37°C for 24 h using Bioscreen. The MICs of BS No. 3 were determined based on the definition described in Section 3.2.7. The remainder of each medium was also incubated at 37°C for 24 h and then used to determine MBC. To determine MBC, each medium containing greater than 2.4% BS No. 3 was diluted to a concentration of 2.4% by the addition of TSB. One millilitre of each medium was then pipetted directly onto an SBA plate and the medium distributed with a sterile glass spreader. These plates were incubated at 37°C for 48 h. Colonies growing on the SBA were identified using the *Salmonella* identification procedure. The MBCs of BS No. 3 was determined based on the definition described previously.

Growth I the presence of different concentrations of bile salts:

Media:

TSB media containing graded concentrations of BS No. 3 were prepared for the experiment. The concentration of BS No. 3 in each medium was 0.075, 0.15, 0.3, 0.6, 1.2, 2.4, 4.8 and 9.6% (w/v). A TSB medium containing no BS No. 3 was also prepared as a positive control.

Methods:

Turbidity of each strain in TSB containing different concentrations of BS No. 3 was determined at 37°C for 20 h according to the procedure described previously.

Turbidity of an uninoculated TSB medium was also measured as a negative control. The growth rate during the logarithmic growth phase of each strain was compared.

Results for Example 4:

MIC and MBC determination:

The MICs and MBCs of BS No.3 for each strain are shown in Table 16.

TABLE 16 : MICs AND MBCs OF BS NO. 3 FOR FD436 AND MUTANTS N-RM4, N-RM25 AND R-NM29.

Strain	MIC of BS No. 3 (%)	MBC of BS No. 3 (%)
FD436	19.2	24
N-RM4	19.2	24
N-RM25	14.4	24
R-NM29	14.4	24

Growth in the presence of different concentrations of bile salts:

As shown in Figure 11, all tested strains showed a prolonged lag phase in their growth cycles in the presence of BS No. 3, albeit to different degrees. Results from the twenty-hour incubation showed that, although the degree of inhibition differed for each strain, the higher the concentration of BS No. 3 the slower the growth rate. N-RM4 inoculated in TSB media containing less than 2.4% BS No. 3 produced faster growth than did the positive control after a prolonged lag phase. The growth of N-RM25 in TSB media containing even a very low concentration of BS No. 3 (as low as 0.075%) was much slower than the growth of this strain in a BS free medium. However, the growth of N-RM25 in graded concentrations of BS No. 3 was more uniform and consistent when compared with the other strains. In addition, the growth rate of N-RM25 was markedly slower when compared with the growth rates of other tested strains in TSB media containing the various concentrations of BS No. 3.

EXAMPLE 5

Pathogenicity and virulence of selected vaccine strains in mice

Experimental procedures for Example 5:

Experimental animals:

Mice were prepared following the procedure described previously.

Challenge strains:

FD436 and mutants N-RM4, N-RM25 and R-NM29 were prepared for the experiments following the procedure described previously. Challenge bacteria were prepared following the procedure described previously.

ID_{50} determination:

Study design:

Four groups of forty mice were each divided into eight groups, including a control group to determine the ID_{50} of the bacterial strains (Table 17). Using PBS at 37°C, the prepared bacterial suspension of each strain was subjected to six serial ten-fold dilutions. For the challenge infection, 100 µl aliquots of each dilution, including the original, were administered to mice via intraperitoneal injection. Actual challenge doses are indicated in Table 17. One hundred microlitres of PBS containing no bacteria was administered to the negative control mice in the same manner. Clinical appearance of all mice was observed daily for 5 consecutive days post-inoculation (P.I.). An ID_{50} for each strain was calculated based on the results of this five-day observation.

Observation of clinical appearance:

Five parameters, activity, appetite, dehydration, respiratory rate and faecal consistency, were monitored daily (9:00 a.m.) in order to determine whether the mice suffered from salmonellosis caused by *S. dublin*. Mice showing clinical signs in three or more of the five parameters were classed as being infected by *S. dublin* and immediately euthanased. Heart blood, liver and spleen of the euthanased mice were removed immediately after euthanasia and investigated using the isolation and identification methods for *Salmonella* described previously.

TABLE 17 : CHALLENGE DOSES OF FD436, N-RM4, N-RM25 AND R-NM29 FOR DETERMINING ID_{50} FOR MICE.

FD436		N-RM4		N-RM25		R-NM29	
Group (n)	**Dose (cfu/mouse)**	**Group (n)**	**Dose (cfu/mouse)**	**Group (n)**	**Dose (cfu/mouse)**	**Group (n)**	**Dose (cfu/mouse)**
1 (5)	1.1×10^{8}	1 (5)	1.2×10^{8}	1 (5)	5.1×10^{8}	1 (5)	2.7×10^{8}
2 (5)	1.1×10^{7}	2 (5)	1.2×10^{7}	2 (5)	5.1×10^{7}	2 (5)	2.7×10^{7}
3 (5)	1.1×10^{6}	3 (5)	1.2×10^{6}	3 (5)	5.1×10^{6}	3 (5)	2.7×10^{6}
4 (5)	1.1×10^{5}	4 (5)	1.2×10^{5}	4 (5)	5.1×10^{5}	4 (5)	2.7×10^{5}
5 (5)	1.1×10^{4}	5 (5)	1.2×10^{4}	5 (5)	5.1×10^{4}	5 (5)	2.7×10^{4}
6 (5)	1.1×10^{3}	6 (5)	1.2×10^{3}	6 (5)	5.1×10^{3}	6 (5)	2.7×10^{3}
7 (5)	1.1×10^{2}	7 (5)	1.2×10^{2}	7 (5)	5.1×10^{2}	7 (5)	2.7×10^{2}
Control (5)	0	**Control (5)**	0	**Control (5)**	0	**Control (5)**	0

n = number of mice in each grop; cfu = colony forming unit.

Penetration and pathogenicity:

Study design:

Fifty-four mice were divided into 4 groups and 4 mice placed in a negative control group (Table 18). Each mouse in all groups excluding the negative control group was experimentally infected once with 100 µl of a bacterial suspension containing FD436, N-RM 4, N-RM 25 or R-NM 29 intraperitoneally. The dose of each strain administered to the mice was approximately one-tenth of the ID_{50} of the corresponding strain. One hundred microlitres of PBS containing no bacteria was

administered to the negative control mice in the same manner. Actual doses are indicated in Table 18.

Clinical appearance was monitored daily for 9 consecutive days P.I. for the mice inoculated with FD436, N-RM4 and R-NM29 and 24 consecutive days P.I. for the mice inoculated with N-RM25 in accordance with the method described previously. To investigate faecal shedding of *Salmonella*, up to 10 faecal pellets were collected daily from each group. At set points in time during the experiments (Table 19), two mice in each group were fasted for 4 h, euthanased and immediately autopsied. Bacteriological investigation was conducted on heart blood, gall bladder bile and liver plus spleen samples collected from one of the euthanased mice in each group to evaluate the level of penetration by the inoculated organism. The gall bladder, liver and spleen of the second mouse in each group were subjected to histopathological examination.

Isolation of *Salmonella* from faeces:

Isolation and identification methods described previously were employed to investigate whether or not *Salmonella* was excreted in faeces. Bacteria identified as being *Salmonella* were further examined to determine whether they were nalidixic acid and/or rifampicin resistant using the identification method described previously. As it was difficult to obtain a specific amount of faeces from each mouse every day, quantitative investigation was not undertaken.

TABLE 18 : DOSES FOR EXPERIMENTAL INFECTION WITH FD436, N-RM4, N-RM25 AND R-NM29.

Group	Strain	Number of mice in group	Dose (cfu/mouse)*
1 (Positive control)	FD436	10	4.9×10
2	N-RM4	10	4.6×10^{3}
3	N-RM25	20	3.8×10^{7}
4	R-NM29	10	2.7×10^{7}
5 (Negative control)	—	4	0

* Bacteria in 100 µl of PBS were injected in each mouse by the intraperitoneal route.
cfu = colony forming unit.

TABLE 19 : TIMETABLE FOR EUTHANASIA.

Day / Group	1	3	5	7	9	12	15	18	21	24
1	E	E	E	E	E					
2	E	E	E	E	E					
3	E	E	E	E	E	E	E	E	E	E
4	E	E	E	E	E					
5	E				E					

Day = number of days post-inoculation with *S. dublin* strains; E = days on which two mice in each group were euthanased.

Isolation of *Salmonella* from heart blood and gall bladder bile:

To detect the presence of *Salmonella*, approximately 20 µl of heart blood and 10 µl of gall bladder bile from one of the euthanased mice in each group were aseptically collected immediately after euthanasia using a 1 ml tuberculin syringe and a 26G×1/2'' needle. Prior to the collection of heart blood, the surface of the heart was sterilised by cauterisation. The surface of the gall bladder was sterilised with 70% ethyl alcohol and then dried immediately before the collection of bile samples. Subsequent *Salmonella* isolation and identification procedures including nalidixic acid and/or rifampicin resistant strains were identical to those outlined previously. Similarly, because it was difficult to obtain a specific amount of heart blood or gall bladder bile from each mouse, quantitative investigation was not undertaken.

Quantitative and qualitative isolation of *Salmonella* from liver plus spleen:

The liver plus spleen samples were placed in boiling water for 3 seconds to sterilise the surface of the organs before culturing. Samples from each mouse were weighed, homogenised together and mixed with PBS to make 5 ml suspensions. The enumeration method described previously was then used to determine the number of bacteria in each sample. The bacteria were subsequently identified as *Salmonella* or otherwise using the *Salmonella* identification method. Any samples that were *Salmonella*-negative in quantitative culturing were subjected to enrichment for qualitative *Salmonella* isolation using the EI methods described previously. Identification procedures for *Salmonella* and for nalidixic acid and/or rifampicin resistant *Salmonella* strains were identical to those outlined previously.

Histopathological investigation of liver, spleen and gall bladder:

Approximately 5 mm by 5 mm tissue samples from liver and spleen, and the entire gall bladder were removed from the mice and fixed in 10% formalin immediately after euthanasia. The tissue samples were processed by conventional means for histopathology. The sections were stained with hematoxylin and eosin (HE) and Giemsa stain, and examined to:

1. Assess lesions, suspected of being caused by inoculated organisms, in the liver, the spleen and the gall bladder and;
2. Evaluate the level of penetration of the gall bladder by the inoculated organisms.

To compare the degree of pathological changes between each inoculated strain, the results of these histopathological investigations were scored using the following scoring systems.

1. Histopathological (HP) score: Based on the level of inflammation, the histopathological changes in the liver spleen and gall bladder were individually scored on a scale of 0 to 3 (0 = normal / no detectable change, 1 = mild changes, 2 = moderate changes, 3 = marked changes – see Table 20

for descriptors of histopathological changes, see Table 27 for HP scores and Tables 28, 29 and 30 for details).

2. Bacterial penetration (BP) score: The levels of penetration of the gall bladder by the organisms were scored on a scale of 0 to 3 (0 = no penetration, 1 = penetration of the lumen only, 2 = penetration of mucosa, 3 = penetration of submucosa – see Table 27 for BP scores and Table 30 for details).

Statistical analysis:

The numbers of mice in each group confirmed as healthy and as ill due to salmonellosis were recorded each day throughout the observation periods. An analysis using Fisher's exact test (two-tailed) was performed on these data to determine whether there were significant differences in the health of mice in the groups when each strain was administered in doses of approximately ten times less than their respective ID_{50}.

TABLE 20 : DESCRIPTORS OF HISTOPATHOLOGICAL CHANGES

Degree	Histopathological changes	
	Liver & spleen	Gall bladder
Normal	Nil	Nil
Mild	A few suppurative foci and/or mild inflammatory infiltrate.	One or more of the following changes: mild epithelial hyperplasia, mild oedema of LP, margination of inflammatory cells and mild inflammatory infiltrate.
Moderate	Moderate degree of one or more of the above changes.	Moderate degree of one or more of the above changes.
Marked	Marked degree of one or more of the above changes and/or other severe changes such as vasculitis and coagulative necrosis.	Marked degree of one or more of the above changes and/or other severe changes such as extension of inflammation to subserosa.

LP = Lamina propria.

Results for Example 5:

ID_{50} determination:

After challenge infection, mice suffering from salmonellosis were found in all groups inoculated with FD436 or N-RM4. Clinical signs shown by mice in these groups included low activity, low appetite, dehydration and high respiratory rates, but not abnormal faecal consistency. None of the mice administered N-RM25 or R-NM29 showed signs of salmonellosis throughout the observation period. Based on the results of clinical obserbations, the ID_{50} for each strain was calculated to be 7.3×10^2 cfu for FD436, 6.9×10^3 cfu for N-RM4, $>5.1\times10^8$ cfu for N-RM25 and $>2.7\times10^8$ cfu for R-NM29. Details are shown in Tables 21 - 24.

TABLE 21 : FIVE-DAY OBSERVATION OF CLINICAL APPEARANCE OF MICE FOLLOWING EXPERIMENTAL INFECTION WITH DIFFERENT DOSES OF FD436.

Group[1] (dose)[2]	Number of mice determined as being infected by *S. dublin*				
	Day[3] 1	Day 2	Day 3	Day 4	Day 5
1 (1.1×10^8)	0	0	5	—	—
2 (1.1×10^7)	0	0	5	—	—
3 (1.1×10^6)	0	0	2	3	—
4 (1.1×10^5)	0	0	2	3	—
5 (1.1×10^4)	0	0	0	2	1
6 (1.1×10^3)	0	0	0	1	2
7 (1.1×10^2)	0	0	0	0	2
Control (0)	0	0	0	0	0

[1]: The number of mice in each group was 5; [2]: cfu; [3]: number of days post-inoculation with the corresponding strains.

TABLE 22 : FIVE-DAY OBSERVATION OF CLINICAL APPEARANCE OF MICE FOLLOWING EXPERIMENTAL INFECTION WITH DIFFERENT DOSES OF N-RM4.

Group[1] (dose)[2]	Number of mice determined as being infected by *S. dublin*				
	Day[3] 1	Day 2	Day 3	Day 4	Day 5
1 (1.2×10^{8})	0	0	1	4	—
2 (1.2×10^{7})	0	0	0	3	2
3 (1.2×10^{6})	0	0	0	2	3
4 (1.2×10^{5})	0	0	0	1	3
5 (1.2×10^{4})	0	0	0	0	2
6 (1.2×10^{3})	0	0	0	0	2
7 (1.2×10^{2})	0	0	0	0	1
Control (0)	0	0	0	0	0

[1]: The number of mice in each group was 5; [2]: cfu; [3]: number of days post-inoculation with the corresponding strains.

TABLE 23 : FIVE-DAY OBSERVATION OF CLINICAL APPEARANCE OF MICE FOLLOWING EXPERIMENTAL INFECTION WITH DIFFERENT DOSES OF N-RM25.

Group[1] (dose)[2]	Number of mice determined as being infected by *S. dublin*				
	Day[3] 1	Day 2	Day 3	Day 4	Day 5
1 (5.1×10^{8})	0	0	0	0	0
2 (5.1×10^{7})	0	0	0	0	0
3 (5.1×10^{6})	0	0	0	0	0
4 (5.1×10^{5})	0	0	0	0	0
5 (5.1×10^{4})	0	0	0	0	0
6 (5.1×10^{3})	0	0	0	0	0
7 (5.1×10^{2})	0	0	0	0	0
Control (0)	0	0	0	0	0

[1]: The number of mice in each group was 5; [2]: cfu; [3]: number of days post-inoculation with the corresponding strains.

TABLE 24 : FIVE-DAY OBSERVATION OF CLINICAL APPEARANCE OF MICE FOLLOWING EXPERIMENTAL INFECTION WITH DIFFERENT DOSES OF N-RM29.

Group[1] (dose)[2]	Number of mice determined as being infected by *S. dublin*				
	Day[3] 1	Day 2	Day 3	Day 4	Day 5
1 (2.7×10^8)	0	0	0	0	0
2 (2.7×10^7)	0	0	0	0	0
3 (2.7×10^6)	0	0	0	0	0
4 (2.7×10^5)	0	0	0	0	0
5 (2.7×10^4)	0	0	0	0	0
6 (2.7×10^3)	0	0	0	0	0
7 (2.7×10^2)	0	0	0	0	0
Control (0)	0	0	0	0	0

[1]: The number of mice in each group was 5; [2]: cfu; [3]: number of days post-inoculation with the corresponding strains.

Penetration and pathogenicity:

Observation of clinical appearance:

As indicated in Table 25, in Group 1 (positive control inoculated with FD436), two mice showed clinical signs of salmonellosis on Day 3 P.I. By Day 5, all surviving mice (6 in total) became ill and two died on that day. The remaining mice died the next day. All mice remaining in Group 2 (inoculated with N-RM4) showed clinical signs of salmonellosis on Day 4. This condition continued and one mouse died on Day 7. The last mice in Group 2 died on Day 8. Clinical signs shown by mice in these groups included low activity, low appetite, dehydration and high respiratory rates. Meanwhilst, all mice in Group 3 (inoculated with N-RM25), Group 4 (inoculated with R-NM29) and Group 5 (negative control) showed no signs of illness throughout the observation period, except 2 mice in group 4 which were ill on Day 5 and then recovered. Statistics show that compared with mice in Groups 1 and 2, mice in Groups 3, 4 and 5 were significantly healthier (between Group 1 and Group 3: $p < 0.0001$, Group 1 and Group 4: $p < 0.001$, Group 1 and Group 5: $p < 0.005$, Group 2 and Group 3: $p < 0.0001$, Group 2 and Group 4: $p < 0.0001$, Group 2 and Group 5: $p < 0.0005$). There was no significant difference in the health of mice between Group 1 and Group 2, nor between Groups 3, 4 and 5.

Isolation of Salmonella from faeces:

Mice in Groups 1 and 2 first excreted *Salmonella* in their faeces on Day 3 and Day 4 P.I., respectively. *Salmonella* were consistently isolated using the DI method until all mice in Groups 1 and 2 were euthanased or died. Throughout the twenty-four day observation period, *Salmonella* was isolated from the faeces of mice in Group 3 intermittently between Day 5 and Day 12, and only using the EI method. On all other days, the mice were culture negative. *Salmonella* was not isolated from the faeces of mice in Groups 4 and 5 during the nine-day observation period. All *Salmonella* isolates from mice in Groups 2 and 3 were nalidixic acid and rifampicin resistant (Table 25).

TABLE 25 : CHRONOLOGICAL INVESTIGATION OF CLINICAL APPEARANCE OF AND *Salmonella* FAECAL EXCRETION BY MICE IN GROUPS INOCULATED WITH FD436, N-RM4, N-RM25 OR R-NM29 VIA THE INTRAPERITONEAL ROUTE.

Group[1]	Day[2]	1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-24	Total
	Total of ill mice/Total of surviving mice[3]	2/26	10/10 (4)[6]	ND[5]						12/36
	Faecal excretion[4] DI	1S/3	2S/2	ND						3S/5
	Faecal excretion[4] EI	1S/3	2S/2	ND						3S/5
	Total of ill mice/Total of surviving mice	0/26	16/16	3/3 (2)[7]						9/45
	Faecal excretion DI	0/3	3R/3	1R/1						4R/7
	Faecal excretion EI	0/3	3R/3	1R/1						4R/7
	Total of ill mice/Total of surviving mice	0/56	0/46	0/38	0/30	0/24	0/18	0/12	0/6	0/230*
	Faecal excretion DI	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/24
	Faecal excretion EI	0/3	2R/3	2R/3	2R/3	0/3	0/3	0/3	0/3	6R/24
	Total of ill mice/Total of surviving mice	0/26	2/16	0/8						2/50*
	Faecal excretion DI	0/3	0/3	0/3						0/9
	Faecal excretion EI	0/3	0/3	0/3						0/9
	Total of ill mice/Total of surviving mice	0/8	0/6	0/6						0/20*
	Faecal excretion DI	0/3	0/3	0/3						0/9
	Faecal excretion EI	0/3	0/3	0/3						0/9

[1]: *Salmonella* strains inoculated to mice were: Group 1 (positive control) - FD436; Group 2 - N-RM4; Group 3 – N-RM25; Group 4 - R-NM29; Group 5 – negative control. Refer to Table 18 for infectious doses.

[2]: Day post-inoculation.

[3]: Two mice in each group were euthanased following the timetable indicated in Table 19.

[4]: Results represent the number of culture positive for *Salmonella* over the total number of samples tested in each group. DI: *Salmonella* was isolated using the direct isolation method, EI: *Salmonella* was isolated using the enrichment isolation method, S: isolated *Salmonella* was sensitive to nalidixic acid and rifampicin, R: isolated *Salmonella* was resistant to nalidixic acid and rifampicin.

[5]: No data were available as no mice survived after Day 6.

[6] & [7]: Numbers in parentheses indicate the number of mice which died during the periods in question ([6]: two mice died on both Days 5 and 6 P.I., [7]: one mouse died on both Days 7 and 8 P.I.).

*: In comparison with Group 1 (positive control), Group 3 $p < 0.0001$, Group 4: $p = 0.0006$, Group 5: $p = 0.0046$ (Fisher's exact test (two-tailed)).

Isolation of *Salmonella* from heart blood:

As shown in Table 26, *Salmonella* was first isolated from heart blood of euthanased mice in all groups, except Group 5, on the first day following inoculation. The organism was isolated from all heart blood samples of mice in Groups 1 and 2 using the DI method until all mice in these groups were euthanased or died. The organism was isolated on Day 3 P.I. from heart blood samples of mice in Group 3 with the DI method. From this point on, the organism was only isolated from a heart blood sample of one mouse in Group 3 using the EI method on Day 7. The organism was not isolated from heart blood samples of mice in Group 4 during the nine-day observation period except on Day 1. No bacteria were isolated from the heart blood samples of mice in Group 5.

Isolation of *Salmonella* from gall bladder bile:

Salmonella was first isolated from gall bladder bile samples of mice in Groups 1, 2 and 3 using the DI method on Day 5 P.I. Afterwards, the organism was isolated from gall bladder bile samples of mice in Group 2 through the DI method on Day 7 (by which time all mice in this group were euthanased or had died). During the twenty-four day observation period, the organism was also isolated from gall bladder bile of mice in Group 3 through the DI method on Day 12 and Day 15. *Salmonella* was also isolated on Day 21 P.I. but only when the EI method was used. The organism was not isolated from gall bladder bile of mice in Groups 4 and 5 (Table 26).

Quantitative and qualitative isolation of *Salmonella* from liver plus spleen:

Salmonella was isolated quantitatively from the livers and spleens of mice in Groups 1, 2 and 3 continuously throughout the observation period (Figure 12). The number of *Salmonella* isolated from the organs of mice in Groups 1 and 2 increased from approximately 10 to approximately 10^7 cfu per gram within 5 days P.I. In contrast, although the dose of N-RM25 administered to mice in Group 3 was approximately 10^4 to 10^6 times greater than that of FD436 and N-RM4, the number

of *Salmonella* isolated from the livers and spleens of mice in Group 3 decreased from approximately 10^3 to approximately 10 cfu per gram by Day 12 P.I. The number of *Salmonella* in the livers and spleens of mice in Group 3 then remained stable at around 10 cfu per gram between Day 12 and Day 24. No bacteria, including *Salmonella*, were isolated from the livers and spleens of mice in Groups 4 and 5 using either direct or enrichment methods (Table 17).

TABLE 26 : CHRONOLOGICAL INVESTIGATION OF THE ISOLATION OF *Salmonella* FROM HEART BLOOD, GALL BLADDER BILE AND LIVER PLUS SPLEEN OF MICE INOCULATED WITH FD436, N-RM4, N-RM25 OR R-NM29 VIA THE INTRAPERITONEAL ROUTE.

Day[1] GROUP[2]	SAMPLE[3]	1	3	5	7	9	12	15	18	21	24
1	HB[4]	+S	+S	+S	ND	ND					
	GB	–	–	+S	ND	ND					
	L&S	+S	+S	+S	ND	ND					
2	HB	+R	+R	+R	+R	ND					
	GB	–	–	+R	+R	ND					
	L&S	+R	+R	+R	+R	ND					
3	HB	+R	+R	–	+RE	–	–	–	–	–	–
	GB	–	–	+R	–	–	+R	+R	–	+RE	–
	L&S	+R	+R	+R	+R	+R	+R	+R	+R	+R	+R
4	HB	+R	–	–	–	–					
	GB	–	–	–	–	–					
	L&S	–	–	–	–	–					
5	HB	–	ND	ND	ND	–					
	GB	–	ND	ND	ND	–					
	L&S	–	ND	ND	ND	–					

[1]: Day post-inoculation.
[2]: *Salmonella* strains inoculated to mice were: Group 1 (positive control) - FD436; Group 2 - N-RM4; Group 3 – N-RM25, Group 4 - R-NM29; Group 5 – negative control. Refer to Table 18 for infectious doses.
[3]: HB – heart blood, GB – gall bladder bile, L&S – liver plus spleen.
[4]: +: positive for *Salmonella* isolation by using direct isolation method, –: negative for *Salmonella* isolation, S: *Salmonella* isolated were sensitive to nalidixic acid and rifampicin, R: *Salmonella* isolated were resistant to nalidixic acid and rifampicin, E: positive for *Salmonella* isolation only by using enrichment isolation method, ND: no data available as mice either did not survive or were not euthanased.

Autopsy:

No gross changes were evident in the organs of mice euthanased on the day following inoculation. In Group 1, scattered petechial haemorrhages were observed on the serosa of the liver in mice euthanased on Day 3 P.I. Cloudy, straw-coloured fibrinous exudate appeared on the surface of the serosa of the visceral organs and the peritoneum of mice in Group 1 euthanased on Day 5. Splenomegaly and petechial haemorrhages on the serosa of the visceral organs (especially the liver) were also prominent in the same mice. In mice in Group 2 euthanased on Day 5, petechial haemorrhages scattered on the entire surface of the liver and the intestine and splenomegaly were observed. One of the mice also showed marked fibrinous exudate in the abdominal cavity. Prominent splenomegaly, petechial haemorrhages on the serosa of the visceral organs and fibrinous exudate on the surface of the visceral organs and peritoneum were observed in mice in Group 2 euthanased on Day 7. There were small (up to miliary size) grey-white foci scattered across the entire surface of the liver of one of these mice (Figures 13A and 13B). In contrast to these findings, no gross lesions were observed upon autopsy of the mice in Group 3 throughout the observation period with the exception of slight to moderate splenomegaly in some mice euthanased on Days 5, 7, 9 and 12 (Figure 14). No lesions were observed in visceral organs in mice in Groups 4 and 5 throughout the observation period.

Histopathological investigation of liver, spleen and gall bladder:

Group 1:

A few foci of mild inflammatory infiltrate characterised by neutrophils and lymphocytes were observed in the liver of the mouse euthanased on the day following inoculation. In this organ, there was widespread infiltration by PMNs and randomly distributed foci of lytic necrosis with suppuration on Day 3 P.I. (Figure 15) followed by an increase in infiltration of the sites by macrophages on Day 5. Vasculitis of portal vessels and coagulative necrosis were also evident. Putative *Salmonella* cells were observed predominantly in Kupffer cells on Day 5 (Figure

16). All mice in this group surviving until Day 5 subsequently died the following day.

Compared with the liver samples, the progress of inflammation in the spleen of mice in this group was somewhat slower. A moderate inflammation in the sinus areas associated with sinusoidal hypercellularity was found on Day 3 P.I. There was a prominent increase in the infiltration by PMNs to these sites on Day 5.

In the gall bladder, obvious changes in histopathology were not observed until Day 3 P.I. Marked epithelial hyperplasia, infiltration by lymphocytes and monocytes, and oedema of the lamina propria were evident on Day 5 (Figure 21). Prominent penetration by bacteria of the lumen, mucosa and submucosa in the gall bladder was also observed on the same day (Figure 22).

Group 2:

As observed in Group 1, one mouse in this group euthanased on Day 3 also showed marked histopathological changes in the liver, such as randomly distributed foci characterised by active infiltration by PMNs. Vasculitis of portal vessels and coagulative necrosis were prominent and putative *Salmonella* cells were predominantly observed in Kupffer cells on Day 5 (Figure 16). A change in predominant cell types from PMNs to lymphocytes and plasma cells between Day 5 and Day 7 was evident. All mice in this group died by Day 8.

Acute splenitis was evident from the active infiltration by PMNs in the spleen of a mouse euthanased on Day 3 (Figure 18). Lytic necrosis in sinus areas and vasculitis accompanied by vascular thrombosis were prominent on Day 5. A change in the types of leukocyte in the sites from PMNs to mononuclear (MN) cells, such as macrophages occurred on Day 7.

In the gall bladder, mild infiltration of the lamina propria by PMNs and MN cells, and bacterial penetration of the lumen were observed on Day 5. The extension of

inflammation from the mucosa to subserosa and the progression to severe acute cholecystitis were evident in a mouse euthanased on Day 7. This was characterised by prominent infiltration by PMNs throughout the gall bladder wall, marked epithelial hyperplasia, haemorrhage and fibroplasia in the lamina propria, and subserosal oedema and fibroplasia (Figure 23A). Marked intraluminal inflammatory exudate by PMNs, monocytes and lymphocytes was also seen (Figure 23B). Numerous bacteria were present in the lumen and adhering to the brush border. Some organisms were also observed in the epithelial cells and submucosa (Figure 24).

Group 3:

Compared with the above two groups, histopathological changes in the liver of mice in this group throughout the observation period were mild. Mild inflammation, such as periportal infiltration by MN cells and a few to a moderate number of foci of neutrophils, was seen between Day 3 and Day 12. During the same period, the number of suppurative foci markedly decreased (mainly PMNs were present in the early stages, after which they were gradually replaced by lymphocytes). Mild inflammation characterised by periportal inflammatory infiltrate and occasional foci continued throughout the twenty-four day observation period (Figure 17). Differing from Groups 1 and 2, prominent hepatocellular regeneration was evident in the liver of mice in this Group euthanased after Day 7 P.I.

In the spleen, mild inflammation characterised by lymphoid hyperplasia, some foci with suppuration in sinus areas (although fewer in number than in mice in Groups 1 and 2) and extramedullary haematopoiesis were observed between Day 5 and Day 9. Apparent inflammation was no longer evident after Day 12 (Figure 19).

Mice in this group showed markedly slower progression of inflammation in the gall bladder than did mice in Groups 1 and 2, even though *Salmonella* was first isolated from this organ in mice in Groups 1, 2 and 3 on the same day (Day 5 P.I.). A mild

but obvious inflammation was first observed in the gall bladder of one mouse in Group 3 euthanased on Day 5 (Figure 25). Inflammation sites in which MN cells were predominant were restricted to the lamina propria. However, inflammation was not evident from the gall bladders of mice euthanased on Day 7 and Day 9. Moderate inflammation characterised by enlargement of epithelial cells and mild oedema of the lamina propria was observed on Day 12. Increases in the number of PMNs infiltrating these sites were also seen on Days 12 and 15. Very mild or no apparent inflammation was observed in the gall bladders during the rest of the observation period (Figure 26). Whilst a small number of putative *Salmonella* cells were intermittently observed in the gall bladder lumen during the twenty-four day observation period, penetration by the organism of the mucosa or deeper sites of the organ was not evident.

TABLE 27 : HISTOPATHOLOGICAL (HP) AND BACTERIAL PENETRATION (BP) SCORES

Score	Day[3] / Group		1	3	5	7	9	12	15	18	21	24
HP score[1]	1	Liver	1	3	3	ND[4]	ND					
		Spleen	0	2	3	ND	ND					
		Gall bladder	0	1	3	ND	ND					
	2	Liver	0	3	3	2	ND					
		Spleen	0	2	3	2	ND					
		Gall bladder	0	0	1	3	ND					
	3	Liver	0	1	2	2	2	1	1	1	1	1
		Spleen	0	0	2	1	1	0	0	0	0	0
		Gall bladder	0	0	1	0	0	2	2	0	1	0
BP score[2]	1		0	0	3	ND	ND					
	2		0	0	1	3	ND					
	3		0	0	1	0	0	1	1	0	1	0

[1]: 0 = no detectable change, 1 = mild change, 2 = moderate change, 3 = marked change.
[2]: 0 = no penetration, 1 = penetration of the lumen only, 2 = penetration of the mucosa, 3 = penetration of the submucosa.
[3]: day post-inoculation,
[4]: no data available as no mice survived.

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TABLE 28 : CHRONOLOGICAL CHANGE IN HISTOPATHOLOGY OF THE LIVER OF MICE INOCULATED WITH FD436, N-RM4, N-RM25 OR R-NM29 VIA INTRAPERITONEAL ROUTE.

Day[1] / Group[2]	Day 1		Day 3		Day 5	
	Inflammation[3]	Score[4]	Inflammation	Score	Inflammation	Score
1	D: a very few foci of inflammatory cells. C: PMNs, lymphocytes.	1	D: randomly distributed suppurative foci. C: PMNs (predominant). R: focal hepatitis, sinusoidal hypercellularity, increase in the number of Kupffer cells, coagulative necrosis.	3	D: randomly distributed suppurative foci. C: PMNs, macrophages. R: hepatitis, coagulative necrosis, vasculitis of portal vessels.	3
2	*Nil*	0	D: randomly distributed suppurative foci. C: PMNs, macrophages. R: focal hepatitis, coagulative necrosis, sinusoidal hypercellularity.	3	D: randomly distributed suppurative foci. C: PMNs. R: hepatitis, vasculitis of portal vessels.	3
3	*Nil*	0	D: a few suppurative foci and mild periportal MN cell infiltrate. C: PMNs, lymphocytes, ± macrophages .	1	D: randomly scattered suppurative foci but mild periportal MN cell infiltrate. C: foci – PMNs (predominant) Periportal – MN cells.	2
5	*Nil*	0	*ND*	ND	ND	ND

Table 28 (cont'd.)

Day Group	Day 7 Inflammation	Day 7 Score	Day 9 Inflammation	Day 9 Score	Day 12 Inflammation	Day 12 Score
1	ND	ND	ND	ND		
2	D: scattered supparative foci, sinusoidal leukocytosis (periportal and portal) C: lymphocytes, plasma cells, macrophages, scattered PMNs. R: prominent extramedullary haematopoiesis.	2	ND	ND		
3	D: scattered suppurative foci and moderate periportal inflammatory infiltrate. C: foci – PMNs, lymphocytes Periportal– MN cells, ± PMNs R: prominent extramedullary haematopoiesis and hepatocellular regeneration.	2	D: scattered suppurative foci and moderate periportal inflammatory infiltrate. C: foci - PMNs, lymphocytes Periportal – lymphocytes, plasma cells, macrophages. R: prominent sinusoidal leukocytosis, extramedullary haematopoiesis and hepatocellular regeneration..	2	D: a few suppurative foci and moderate periportal inflammatory infiltrate C: foci- lymphocytes, ± PMNs periportal→lymphocytes, plasma cells, ± PMNs. R: sinusoidal hypercellularity, extramedullary haematopoiesis.	1
5	ND	ND	Nil	0		

Table 28 (cont'd)

Day / Group	Day 15		Day 18		Day 21	
	Inflammation	Score	Inflammation	Score	Inflammation	Score
3	D: mild periportal inflammatory infiltrate and very occasional suppurative foci. C: PMNs, lymphocytes, plasma cells. R: extramedullary haematopoiesis and sinusoidal hypercellularity.	1	D: mild periportal inflammatory infiltrate and very occasional, random suppurative foci. C: PMNs, lymphocytes, plasma cells, macrophages. R: extramedullary haematopoiesis.	1	D: mild periportal inflammatory infiltrate and very occasional, random suppurative foci. C: lymphocytes, plasma cells, macrophages, ± PMNs. R: sinusoidal hypercellularity.	1

Table 28 (cont'd)

Day / Group	Day 24	
	Inflammation	Score
3	D: mild periportal inflammatory infiltrate. C: lymphocytes, plasma cells, macrophages, ± PMNs.	1

[1]: Day post-inoculation.
[2]: Mice in each group were intraperitoneally inoculated with the following *Salmonella* strains; Group 1: FD436; Group 2: N-RM4; Group 3: N-RM25 and Group 4: R-NM29.
[3]: D: distribution, C: cell types, R: remarks, PMN: polymorphonuclear neutrophil granulocyte, MN: mononuclear, ±: a small number of, Nil: no histopathological changes were observed, ND: no data available as mice either did not survive or were not euthanased.
[4]: Score: histopathological score.

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TABLE 29 : CHRONOLOGICAL CHANGE IN HISTOPATHOLOGY OF THE SPLEEN OF MICE INOCULATED WITH FD436, N-RM4, N-RM25 OR R-NM29 VIA INTRAPERITONEAL ROUTE

Group[2]	Day 1		Day 3		Day 5	
	Inflammation[3]	Score[4]	Inflammation	core	Inflammation	Score
1	Nil	0	D: scattered suppurative foci in sinus areas. C: macrophages, ± PMNs. R: sinusoidal hypercellularity, prominent extramedullary haematopoiesis.	2	D: scattered suppurative foci in sinus areas. C: macrophages, scattered and clustered PMNs. R: splenitis, necrosis of some foci, sinusoidal hypercellularity, prominent extramedullary haematopoiesis.	3
2	Nil	0	D: scattered suppurative foci in sinus areas. C: macrophages, scattered and clustered PMNs. R: sinusoidal hypercellularity.		D: scattered suppurative foci in sinus areas. C: macrophages, scattered and clustered PMNs. R: splenitis, lytic necrosis in sinus areas, vascular thrombosis.	3
3	Nil	0	R: very little inflammation – no apparent phagocytic action.	0	D: scattered suppurative foci in sinus areas. C: macrophages, ± PMNs. R: sinusoidal hypercellularity, prominent extramedullary haematopoiesis	
5	Nil	0	ND	ND	ND	D

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Table 29(cont'd.)

Day Group	Day 7		Day 9		Day 12	
	Inflammation	core	Inflammation	core	Inflammation	core
1	ND	ND	ND	ND		
2	D: scattered suppurative foci in sinus areas. C: macrophages, scattered lymphocytes, plasma cells. R: very few PMNs, prominent extramedullary haematopoiesis.	2	ND	ND		
3	D: occasional random foci of inflammatory cells in sinus areas. C: sinus – macrophages. foci – PMNs. R: prominent extramedullary haematopoiesis.		R: a little number of lymphocytes in sinusoids, moderate lymphoid hyperplasia.		R: moderate lymphoid hyperplasia.	0
5	ND	ND	Nil	0		

Table 29 (cont'd)

Day Group	Day 15		Day 18		Day 21	
	Inflammation	Score	Inflammation	Score	Inflammation	core
3	R: mild lymphoid hyperplasia	0	R: mild lymphoid hyperplasia.	0	R: mild lymphoid hyperplasia.	0

Table 29 (cont'd)

Day Group	Day 24	
	Inflammation	Score
3	R: mild lymphoid hyperplasia.	0

[1]: Day post-inoculation.
[2]: Mice in each group were intraperitoneally inoculated with the following *Salmonella* strains; Group 1: FD436; Group 2: N-RM4; Group 3: N-RM25 and Group 4: R-NM29.
[3]: D: distribution, C: cell types, R: remarks, PMN: polymorphonuclear neutrophil granulocyte, MN: mononuclear, ±: a small number of, Nil: no histopathological changes were observed, ND: no data available as mice either did not survive or were not euthanased.
[4]: Score: histopathological score.

TABLE 30 : CHRONOLOGICAL CHANGE IN HISTOPATHOLOGY AND *SALMONELLA* PENETRATION OF THE GALL BLADDER OF MICE INOCULATED WITH FD436, N-RM4, N-RM25 OR R-NM29 VIA INTRAPERITONEAL ROUTE

Day[1] / Group[2]	Day 1				Day 3			
	Inflammation[3]	Score 1[4]	Penetration[5]	Score 2[4]	Inflammation	Score 1	Penetration	Score 2
1	Nil	0	Nil	0	R: mild oedema of LP.	1	Nil	0
2	Nil	0	Nil	0	Nil	0	Nil	0
3	Nil	0	Nil	0	Nil	0	Nil	0
5	Nil	0	Nil	0	ND	ND	ND	ND

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Table 30 (cont'd)

Day Group	Day 5				Day 7			
	Inflammation	Score 1	Penetration	Score 2	Inflammation	Score 1	Penetration	Score 2
1	D: LP C: lymphocytes, monocytes, PMNs. R: cholecystitis, marked epithelial hyperplasia, congestion and oedema of LP.	3	Lumen, mucosa, submucosa.	3	ND	ND	ND	ND
2	D: LP. C: PMNs, MN cells. R: mild focal hyperplasia of epithelial cells.	1	Lumen.	1	D: intraepithelial, intraluminal, LP. C: PMNs (70%), MN cells. R: cholecystitis, marked epithelial hyperplasia, mesothelial hyperplasia, oedema & haemorrhage & fibroplasia in LP, subserosal oedema & infiltrate.	3	Lumen, mucosa, submucosa.	3
3	D: LP. C: predominantly MN cells, ± PMNs. R: mild epithelial hyperplasia, congestion of LP, margination in vessels.	1	Lumen.	1	R: very mild epithelial hyperplasia.	0	Nil	0
5	ND	ND	ND	ND	ND	ND	ND	ND

Table 30 (cont'd)

Day Group	Day 9				Day 12			
	Inflammation	Score 1	Penetration	Score 2	Inflammation	Score 1	Penetration	Score 2
1	ND	ND	ND	ND				
2	ND	ND	ND	ND				
3	R: very mild oedema of LP.	0	Nil.	0	D: intraepithelial, LP, subserosal. C: MN cells (70%), PMNs. R: moderate epithelial hyperplasia, mild oedema of LP, subserosal oedema with MN inflammatory infiltrate.	2	Lumen	1
5	Nil	0	Nil	0				

Table 30 (cont'd)

Day Group	Day 15 Inflammation	Score 1	Penetration	Score 2	Day 18 Inflammation	Score 1	Penetration	Score 2
3	D: intraepithelial, LP, subserosal. C: PMNs (50%), MN cells (50%). R: moderate epithelial hyperplasia, mild oedema of LP, subserosal inflammatory infiltrate.	2	Lumen.	1	R: very mild MN inflammatory infiltrate in LP.	0	Nil	0

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Table 30 (cont'd)

Day / Group	Day 21				Day 24			
	Inflammation	Score 1	Penetration	Score 2	Inflammation	Score 1	Penetration	Score 2
3	D: LP (only a few suppurative foci). C: PMNs (50%), MN cells (50%).	1	Lumen.	1	Nil	0	Nil	0

[1]: Day post-inoculation.
[2]: Mice in each group were intraperitoneally inoculated with the following *Salmonella* strains; Group 1: FD436; Group 2: N-RM4; Group 3: N-RM25 and Group 4: R-NM29.
[3]: D: distribution, C: cell types, R: remarks, PMN: polymorphonuclear neutrophil granulocyte, MN: mononuclear, ±: a small number of, LP: lamina propria, Nil: no histopathological changes were observed, ND: no data available as mice either did not survive or were not euthanased.
[4]: Score 1: histopathological score, Score 2: bacterial penetration score.
[5]: Penetration by inoculated bacteria.

EXAMPLE 6

Vaccine trials in mice

Experimental procedures for Example 6:

Experimental animals:

Mice used in experiments described in this chapter were prepared following the procedure described previously. The number of mice used for each experiment was: 10 for Experiment 1, 35 for Experiment 2 and 18 for Experiment 3. Tables 31, 32 and 33 show the designs adopted to study the three aspects outlined above.

Vaccine and challenge strain preparation:

N-RM25, N-RM4 and R-NM29 were prepared as vaccines and FD436 for challenge infection according to the procedures described previously. Vaccine doses used in the experiments are shown in Tables 31, 32 and 33. The suspension containing FD436 was prepared to a concentration approximately 100 times greater than its ID_{50} for mice. Actual challenge doses for the experiments are indicated in Tables 31, 32 and 33.

Experiment 1 – Vaccine trial using single vaccination with a set dose of N-RM25:

Study design:

Ten mice were divided into 2 groups. Mice in Group 1 were intraperitoneally vaccinated once with 100 µl of the vaccine suspension containing N-RM25. Mice in Group 2 were injected with 100 µl of sterile PBS as a control. Challenge infection with FD436 was carried out on Day 21 post-vaccination (P.V.). Vaccine and challenge doses are shown in Table 31.

The clinical appearance of each mouse was monitored daily for 51 days P.V. (30 days post-challenge (P.C.)) in accordance with the method described previously.

Faecal pellets from each mouse were collected daily during the same period to examine faecal shedding of both vaccine and challenge strains. Mice which showed severe clinical signs of salmonellosis were euthanased promptly and autopsied. Mice that survived throughout the monitoring period were euthanased on Day 52 P.V. and autopsied. Bacteriological investigation was then conducted on heart blood and gall bladder bile samples collected from euthanased mice to isolate *Salmonella*. Bacteriological investigations, including enumeration, were also conducted on the liver and spleen of those mice.

Isolation of *Salmonella* from faeces, heart blood, gall bladder bile and liver plus spleen:

The culture methods for the above samples were as per the methods described previously.

Statistical analysis:

Following challenge infection, statistical comparisons were made using Fisher's exact test (two-tailed) comparing the number of surviving mice and the number of mice euthanased due to clinical signs of salmonellosis between the vaccinated and the control groups to evaluate whether vaccination was efficacious.

The number of mice in each group confirmed to be positive or negative for faecal excretion of *Salmonella* was recorded each day after challenge infection. An analysis using Fisher's exact test (two-tailed) was performed on these data to assess whether the vaccine significantly prevented faecal shedding of the challenge strain.

Experiment 2 – vaccine trials using single and double vaccination with different doses of N-RM25:

Study design:

Thirty-five mice were divided into 7 groups. Mice in Groups 1, 3 and 5 were intraperitoneally vaccinated once with 100 µl of graded concentrations of N-RM25.

Mice in Groups 2, 4 and 6 were vaccinated twice in the same manner. The booster vaccination (B.V.) was administered on Day 21 P.V. Mice in the control group were injected via the intraperitoneal route with 100 µl of sterile PBS twice at the same interval. Challenge infection was carried out using FD436 intraperitoneally on Day 35 P.V. (Day 14 post-booster vaccination (P.B.V.)). Vaccine and challenge doses are shown in Table 32.

The clinical appearance of each mouse was monitored daily until 65 days P.V. Faecal pellets from each mouse were collected daily during the same period for bacteriological investigation. Mice which showed severe clinical signs of salmonellosis were euthanased promptly and autopsied. Mice that survived throughout the monitoring period were euthanased on Day 66 P.V. and autopsied. Bacteriological investigations were conducted on heart blood and gall bladder bile samples collected from the euthanased mice. Bacteriological investigations, including enumeration, were also performed on the liver and spleen. To further investigate the distribution of *Salmonella* in vaccinated mice, the following organ samples were also examined: lung, duodenum and jejunum, ileum, ileocaecum, caecum, colon and kidney.

Isolation of *Salmonella* from faeces, heart blood, gall bladder bile and liver plus spleen:

The culture methods for the above samples were as per the methods described previously.

Isolation of *Salmonella* from organ samples:

Organ samples (lung, duodenum and jejunum, ileum, ileocaecum, caecum, colon and kidney) were placed in boiling water for 3 seconds to sterilise the surface of the organs. Duodenum and jejunum, ileum, ileocaecum, caecum and colon samples were then cut and opened out with a scalpel. Gut contents were gently rinsed from the samples using sterile PBS. Each sample was homogenised and processed for isolation of *Salmonella*. Isolates identified as *Salmonella* were further investigated

to determine their resistance to nalidixic acid and rifampicin. Bacteriological investigation of the organs was performed only on mice which did not yield *Salmonella* from a heart blood culture using the DI method due to the difficulty in distinguishing between actual colonisation of the organs by the organism and the temporary presence of the organism in the organs via blood circulation.

Statistical analysis:

Following challenge infection, statistical comparisons were made using Fisher's exact test (two-tailed) comparing the number of surviving mice and the number of mice euthanased due to clinical signs of salmonellosis between each of the vaccinated groups and the control. This was done to evaluate the protective properties of N-RM25 administered using different vaccination regimes and doses. When statistically insignificant results were obtained from the above analysis, time periods until death (the time period from challenge infection to euthanasia due to clinical signs of salmonellosis) were also compared using the Student's t test (two-tailed).

The numbers of mice in each group confirmed as positive or negative for faecal excretion of *Salmonella* were recorded daily throughout the observation period. An analysis using Fisher's exact test (two-tailed) was performed on data obtained before challenge infection. This allowed evaluation of which vaccination methods and doses were the most efficient for minimising post-vaccination faecal shedding. Data obtained post-challenge infection was also analysed using the same test to evaluate which vaccination methods and doses were the most effective in preventing faecal shedding of the challenge strain.

Experiment 3 – Vaccine trials using N-RM4 or R-NM29:

Study design:

Eighteen mice were divided into 3 groups. Mice in Group 1 were vaccinated once via the intraperitoneal route with 100 μl of vaccine solution containing N-RM4.

Mice in Group 2 were vaccinated twice with vaccine solution containing R-NM29 in the same manner separated by an interval of 21 days. Mice in Group 3 were intraperitoneally injected twice with 100 µl of sterile PBS at the same interval as a control. Challenge infection was carried out using FD436 via the intraperitoneal route on Day 35 P.V. Vaccine and challenge doses are shown in Table 33.

Clinical appearance of each mouse was monitored daily until 65 days P.V. Faecal pellets from each mouse were collected daily for bacteriological investigation. Mice which showed severe clinical signs of salmonellosis were euthanased promptly and autopsied. Mice that survived throughout the monitoring period were euthanased on Day 66 P.V. and autopsied. Bacteriological investigation was conducted on heart blood and organ samples collected from the euthanased mice. Bacteriological investigations, including enumeration, were also conducted on the liver and spleen.

Isolation of *Salmonella* from faeces, heart blood, gall bladder bile and liver plus spleen:

The culture procedures for the above samples were the same as those described previously.

Isolation of *Salmonella* from organ samples:

The culture method for the samples followed the procedure described previously.

TABLE 31 : *S. dublin* VACCINE TRIAL IN MICE USING SINGLE INTRAPERITONEAL VACCINATION WITH A SET DOSE OF N-RM25.

Group	Number of mice	Vaccine dose[1] (cfu/mouse)	Challenge strain[2]	Challenge dose (cfu/mouse)
1	5	2.4×10^{8}	FD436	4.7×10^{4}
2 (control)	5	0[3]	FD436	4.7×10^{4}

[1]: Vaccination and challenge infection were administered via the intraperitoneal route.
[2]: Challenge infection was carried out on Day 21 P.V.
[3]: Each mouse was injected 100 µl of sterile PBS.

TABLE 32 : *S. dublin* VACCINE TRIALS IN MICE USING SINGLE AND DOUBLE INTRAPERITONEAL VACCINATION WITH DIFFERENT DOSES OF N-RM25.

Group		Number of mice	Vaccination method	Vaccination dose (cfu/mouse)	Booster vaccination dose[1] (cfu/mouse)
1	Low[2]	5	Single	1.7×10^3	N/A
2	Low	5	Double	1.7×10^3	1.9×10^4
3	Medium[3]	5	Single	1.7×10^5	N/A
4	Medium	5	Double	1.7×10^5	1.9×10^6
5	High[4]	5	Single	1.7×10^7	N/A
6	High	5	Double	1.7×10^7	1.9×10^8
7 (control)[5]	*N/A*	5	Double	0	0

[1]: The booster vaccination was carried out on Day 21 P.V. and challenge infection was administered via the intraperitoneal riute using FD436 with a dose of 4.6×10^4 cfu/mouse on Day 35 P.V.
[2]: Low: the low vaccine dose group.
[3]: Medium: the medium vaccine dose group.
[4]: High: the high vaccine dose group.
[5]: Mice were injected 100 µl of sterile PBS via the intraperitoneal route.

TABLE 33 : *S. dublin* VACCINE TRIALS IN MICE USING INTRAPERITONEAL VACCINATION WITH N-RM4 OR R-NM29.

Group	Vaccine strain	Number of mice	Vaccination method	Vaccination dose (cfu/mouse)	Booster vaccination dose[1] (cfu/mouse)
1	N-RM4	5	Single	7.5×10^2	N/A
2	R-NM29	8	Double	2.7×10^7	1.7×10^8
3 (control)	N/A	5	Double	0^2	0^2

[1]: The booster vaccination was carried out on Day 21 P.V. and challenge infection was administered via the intraperitoneal route using FD436 with a dose of 7.3×10^4 cfu/mouse on Day 35 P.V.
[2]: Mice were inoculated with 100 µl of sterile PBS via the intraperitoneal route.

Results for Example 5:

Vaccine trial using single vaccination with a set dose of N-RM25:

Observation of clinical appearance:

No clinical symptoms were observed in either vaccinated (Group 1) or control (Group 2) mice before challenge infection. The vaccinated mice did not develop any clinical symptoms following challenge infection. In contrast, all mice except one in the control group developed clinical signs of salmonellosis on Day 2 P.C. All mice in this group were euthanased by Day 5 P.C. due to clinical signs of salmonellosis. The mean time until euthanasia among mice in the control group was 115 h. Therefore, it is clear that the protection conferred by the vaccine was significant ($p = 0.0079$).

Isolation of *Salmonella* from faeces:

Before challenge infection, the vaccine strain was isolated intermittently from the faeces of vaccinated mice from Day 5 to Day 11 P.V. by the EI method only.

Following challenge infection, *Salmonella* was isolated from the faeces of the vaccinated mice significantly less often ($p < 0.0001$) than mice in the control group (Table 34). The vaccinated mice excreted *Salmonella* in the faeces on Day 4 P.C (2 mice), Day 5 (2 mice), Day 7 (3 mice), Day 8 (3 mice), Day 10 (1 mouse) and Day 13 (2 mice) during the 30-day observation period. However, the organism was detectable only by the EI method. These isolates were nalidixic acid and rifampicin sensitive and were identified as the challenge strain (FD436). Compared with this, *Salmonella* was first isolated by the DI method from the faeces of three control mice on Day 2 P.C. It was then isolated from the faeces of the surviving mice continuously until all mice in this group were euthanased (Day 5 P.C.).

Autopsy:

Although there was slight to moderate splenomegaly in two mice in the vaccinated group, no other pathological changes were observed in this group. In contrast, accumulation of cloudy, straw-colour fibrinous exudate, identified as pyogenic exudate, was observed in the abdominal cavity in the control mice. All mice in this group developed splenomegaly and some of them also developed petechial haemorrhages of the serosa of the visceral organs, especially the liver, and the peritoneum. There were small grey-white foci scattered across the entire surface of the livers of two control mice.

Isolation of *Salmonella* from heart blood, gall bladder bile and liver plus spleen:

Salmonella was not isolated from heart blood, gall bladder bile or the liver plus spleen specimens of any of the vaccinated mice. On the other hand, the challenge strain was isolated from all of these specimens from the control mice using the DI method. The mean number of organisms in the liver and spleen of the control mice was 5.0×10^{8} cfu/g (the number ranging between 2.0×10^{6} and 1.0×10^{9} cfu/g) - see Table 34.

TABLE 34 : *S. dublin* VACCINE TRIAL IN MICE USING SINGLE INTRAPERITONEAL VACCINATION WITH A SET DOSE OF N-RM25 FOLLOWED BY CHALLENGE INFECTION WITH WILD STRAIN FD436.

Group[1]		1	2 (control)
Number of surviving Mice[2]	Initial number of mice	5	5
	Pre-challenge	5	5
	Post-challenge	5	0[7]
Mean time until death[3] (h)		N/A	115
Protection (%)		100	0
Faecal *Salmonella* isolation[4] (pre-challenge)		16/100	0/100
Faecal *Salmonella* isolation[4] (post-challenge)		13/150	12/19
Heart blood culturing[5]		0/5	5/5
Gall bladder bile culturing[5]		0/5	5/5
Isolation of *Salmonella* from liver & spleen		0/5	5/5
Mean number of *Salmonella* in the liver & spleen [6] (cfu/g)		0	5.0×10^8

[1]: Vaccine dose for Group 1: 2.4×10^8 cfu/mouse. Group 2 mice received 100 µl of sterile PBS. Challenge dose: 4.7×10^4 cfu/mouse (on Day 21 P.V.)
[2]: Number of surviving mice in the group on Day 20 P.V. (pre-challenge) and Day 51 P.V. (post-challenge).
[3]: For ethical reasons, the time from challenge infection to euthanasia due to critical signs of salmonellosis was used to derive this value.
[4]: Number of cultures positive for the vaccine strain (pre-challenge) and the challenge strain (post-challenge) over the total number of samples tested in each group.
[5]: Number of cultures positive for the challenge strain over the number of samples tested.
[6]: Mean number of the challenge strain organisms isolated from the liver plus spleen samples in each group.
[7]: All mice were euthanased by Day 5 P.C. due to critical signs of salmonellosis.

Vaccine trials using single and double vaccination with different doses of N-RM25:

Observation of clinical appearance:

Prior to challenge infection, no mice showed any clinical symptoms. Following lethal challenge infection, no mice vaccinated with high doses (Groups 5 and 6) showed clinical signs of salmonellosis irrespective of whether the single or the double dose was given. In comparison with this, only one mouse of each group given medium-dose vaccination (Groups 3 and 4) survived throughout the observation period. All mice in the low-dose vaccination groups (Groups 1 and 2)

and the control group developed clinical signs of salmonellosis and were euthanased. Based on the above results, N-RM25 vaccination with high-doses conferred significantly greater protection against challenge infection than vaccination with low and medium doses and the control (high-dose groups vs. medium-dose groups: $p = 0.0007$, vs. low-dose groups and control: $p < 0.0001$). There were no significant differences in protection between the groups receiving low or medium levels of vaccination and the control group (Table 35).

Isolation of *Salmonella* from faeces:

No mice given the low-dose vaccines shed *Salmonella* in the faeces during the pre-challenge period. In contrast, the vaccine strain was isolated from the faeces of some mice in medium and high dose groups during the period. Statistical analysis showed that mice in the double medium-dose group and the high-dose groups excreted the vaccine strain in the faeces significantly more often than mice in both the low-dose group and control group during the period between the first vaccination and challenge infection (low-dose group or control group vs. double medium dose group: $p = 0.0147$, vs. high dose groups: $p < 0.0001$). However, isolation was intermittent and only for short periods (equal to or less than 7 days) following each vaccination and was only possible with EI method. There was no significant difference in the frequency of faecal excretion of the organism between the single vaccination groups and the double vaccination groups.

Following challenge infection, the challenge strain was isolated from the faeces of only two mice in the double high-dose vaccination group (Group 6) (once on Day 9 P.C. from the faeces of one of the two mice, and from the faeces of the other mouse intermittently until 22 days P.C. and then not isolated again during the observation period). These isolates were only obtained using the EI method. The organism was isolated intermittently throughout the observation period from the faeces of three out of five mice in the single high-dose vaccination group (Group 5). Compared with the high-dose groups, the challenge strain was continuously isolated from the

faeces of the mice in the control group using the DI method from Day 2 P.C. to the day on which all mice were euthanased. All mice in the single low-dose group (Group 1) and some in the double low dose group (Group 2) started excreting the strain on Day 3 P.C. and Day 4 P.C., respectively. The strain was then isolated from the faeces of all surviving mice in those groups continuously until the day on which the last mouse in each group was euthanased. Similarly, mice in both the medium vaccination groups first excreted the organism on Day 4 P.C. and surviving mice then did so throughout the observation period. Based on this, following challenge infection, the challenge strain was isolated from faeces of mice in the high-dose groups significantly less often than the faeces of the medium-dose, low-dose and control groups (high-dose groups vs. medium-dose and low-dose groups: $p < 0.0001$, vs. control group: $p = 0.002$). In the high-dose group, furthermore, mice vaccinated with the double dose excreted the strain significantly ($p < 0.0001$) less often than did mice vaccinated with the single dose. There were no significant differences in excretion of the strain between the low-dose and the medium-dose vaccination groups and the control group (Table 35).

Autopsy:

No gross lesions were observed upon autopsy of the mice in the double high-dose group. Three of five mice given the single high-dose vaccine developed only mild pathological changes, such as mild splenomegaly. Compared with the high-dose groups, the surviving mice in the medium-dose groups developed somewhat severer changes, including splenomegaly and a few small, grey-white foci on the surface of the liver and the kidney. On the other hand, twenty-two out of twenty-three mice euthanased due to clinical signs of salmonellosis in the medium-dose and low-dose groups developed from moderate to marked pathological changes. The changes include splenomegaly, petechial haemorrhages on visceral organs, grey-white foci on the surface of the liver and cloudy straw-colour fibrinous exudate in the abdominal cavity. Some euthanased mice also developed grey-white

foci on the surface of the spleen and kidney and grey-white fibrinous exudate in the thoracic cavity.

Isolation of *Salmonella* from heart blood and gall bladder bile:

Salmonella was not isolated even using EI method from heart blood and gall bladder bile of mice in the high-dose groups. In contrast, the challenge strain was isolated from the heart blood of eight out of ten mice in medium-dose groups, all mice in the low-dose and the control groups. All of these mice were euthanased due to clinical signs of salmonellosis. Although gall bladder bile specimens could not be obtained from all of the mice, of the specimens that were taken, the challenge strain was isolated only from those collected from mice euthanased due to clinical sings of salmonellosis (Table 35).

Quantitative and qualitative isolation of *Salmonella* from liver plus spleen:

No *Salmonella* was isolated from liver plus spleen specimens collected from mice given double high-dose vaccination using either quantitative or qualitative methods. The challenge strain was isolated from two mice in the single high-dose group, but only with the qualitative method. In contrast, the strain was isolated quantitatively from mice in the medium-dose and low-dose groups and the control group that were euthanased due to clinical signs of salmonellosis. The numbers of the organism in the organs ranged between 4.9×10^7 and 1.5×10^8, and 3.6×10^6 and 9.3×10^7 cfu/g for single and double medium-dose vaccination groups, respectively, from 1.4×10^7 to 8.8×10^7 for the low-dose groups and between 7.5×10^6 and 1.6×10^8 for the control group (Table 35 and Figure 27). The challenge strain was not isolated from the liver and spleen of mice given medium-dose vaccination that survived challenge. The vaccine strains were not isolated from any liver plus spleen specimen.

Isolation of *Salmonella* from organ samples:

As shown in Table 36, in the double high-dose group, the challenge strain was isolated only from the ileocaecum of one out of five mice using the EI method. Compared with this, the strain was isolated from many organs of mice in the other groups. The strain was isolated from the liver, spleen, ileum, ileocaecum, caecum,

colon and kidney of three of five mice given single high-dose vaccination. The strain was also isolated from the intestine between the ileum and the colon of mice in the medium-dose groups. The vaccine strain was isolated from the ileocaecum and colon of one mouse in the double high-dose group only by using the EI method.

colon and kidney of three of five mice given single high-dose vaccination. The strain was also isolated from the intestine between the ileum and the colon of mice in the medium-dose groups. The vaccine strain was isolated from the ileocaecum and colon of one mouse in the double high-dose group only by using the EI method.

TABLE 35 : *S. dublin* VACCINE TRIALS USING SINGLE AND DOUBLE VACCINATION WITH DIFFERENT DOSES OF N-RM25 FOLLOWED BY CHALLENGE INFECTION WITH WILD STRAIN FD436.

	Group[1]	1	2	3	4	5	6	7 (control)
Number of surviving mice	Initial number of mice	5	5	5	5	5	5	5
	Pre-challenge	5	5	5	5	5	5	5
	Post-challenge	0	0	1	1	5*	5*	0
Mean time until death[2] (h)		115	216	N/A	N/A	N/A	N/A	100
Protection (%)		0	0	20	20	100	100	0
Faecal *Salmonella* isolation[3]	Pre-challenge	0/170	0/170	3/170 (3)[5]	7/170** (7)	14/170** (14)	18/170** (18)	0/170
	Post-challenge	9/24 (0)	23/45 (1)	34/58 (13)	37/66 (20)	52/155 (45)	12/155*** (12)	11/21 (1)
Heart blood culture[4]		5/5	5/5	4/5[6]	4/5[6]	0/5	0/5	5/5
Gall bladder bile culture[4]		1/1	2/2	2/3[6]	1/2[6]	0/5	0/5	4/4
Isolation of *Salmonella* from liver plus spleen[4]		5/5	5/5	4/5[6]	4/5[6]	2/5 (2)	0/5	5/5

[1]: Vaccination: Group 1 = single low-dose, 2 = double low-dose, 3 = single medium-dose, 4 = double medium-dose, 5 = single high-dose, 6 = double high-dose. Booster vaccination: on Day 21 P.V., challenge infection: on Day 35 P.V.. Refer to Table 8.2 for detailed vaccination and challenge infection information.

[2]: For ethical reasons, the time period from challenge infection to euthanasia due to critical illness was used in the calculation of the mean time until death.

[3]: Numbers of cultures positive for the corresponding vaccine strains (pre-challenge) and the challenge strain (post-challenge) over the total number of samples tested in each group.

[4]: Number of cultures positive for the challenge strain over the number of samples tested at autopsy.

[5]: Numbers in parentheses indicate the number of positive results detected only using the EI method.

[6]: All mice that produced negative results survived throughout the observation period.

*: Control vs. $p = 0.0079$; **: control vs. Group 4 $p = 0.0147$, Group 5 $p < 0.0001$, Group 6 $p < 0.0001$; ***: control vs. $p < 0.0001$ (two-tailed Fisher's exact test).

Table 36 : Distribution of *Salmonella* in internal organs of mice that survived throughout vaccine trials using single and double vaccination methods with different doses of N-RM25 followed by challenge infection with wild strain FD436.

Group[1] / Organ	3 (n = 1)	4 (n = 1)	5 (n = 5)	6 (n = 5)
Lung	0/1[2]	0/1	0/5	0/5
Liver & spleen	0/1	0/1	2S/5 (2)	0/5
Duodenum & jejunum	0/1	0/1	0/5	0/5
Ileum	1S/1 (1)[3]	1S/1 (1)	1S/5 (1)	0/5
Ileocaecum	1S/1 (1)	1S/1	3S/5 (2)	1S, 1R/5 (2)
Caecum	0/1	1S/1 (1)	1S/5 (1)	0/5
Colon	1S/1 (1)	1S/1 (1)	3S/5 (2)	1R/5 (1)
Kidney	0/1	0/1	1S/5	0/5

[1]: Vaccination: Group 3 = single medium-dose, 4 = double medium-dose, 5 = single high-dose, 6 = double high-dose. Refer to Table 32 for detailed vaccination and challenge infection information. Organ sample culturing was not performed on mice in Group 1 (single low-dose) and Group 2 (double low-dose) and the control because their heart blood samples were positive for *Salmonella*.

[2]: Results represent the numbers of cultures positive for the vaccine strain and the challenge strain over the total number of samples tested in each group. S: nalidixic acid and rifampicin sensitive (challenge) strain, R: nalidixic acid and rifampicin resistant (vaccine) strain.

[3]: Numbers in parentheses indicate the number of isolates obtained using EI method.

Vaccine trials using N-RM4 or NM29:

All mice vaccinated with N-RM4 showed clinical symptoms following vaccination and one of them died due to acute septicaemia caused by the vaccine strain before challenge infection (Day 8 P.V.). The remaining mice were moderately ill (the mice had shown clinical signs in two or fewer of the five parameters) until Day 12 P.V. then recovered. No other clinical signs were observed in the remaining mice before challenge infection. No mice in this group that survived after vaccination showed clinical symptoms following challenge infection.

Mice receiving R-NM29 and mice in the control group developed no clinical signs before challenge infection (Table 37). Four out of eight mice in the R-NM29 group were euthanased due to clinical signs of salmonellosis on Day 7 P.C. (1 mouse), Day 8 P.C. (2 mice) and Day 12 P.C. (1 mouse). Whilst some of the remaining

mice in this group were moderately ill between Days 5 and 13 P.C., no more mice were euthanased. All mice in the control were euthanased due to clinical signs of salmonellosis by 4 days P.C. (Table 37).

Isolation of *Salmonella* from faeces:

Two mice vaccinated with N-RM4 first excreted this vaccine strain on Day 3 P.V. The strain was then isolated intermittently from mice in this group not only before but also after challenge infection. Mixed isolation of the vaccine strain and the challenge strain was often observed from faecal samples in this group (Table 37).

Salmonella was not isolated from faecal samples collected from the mice in the R-NM29 group before challenge infection. A mouse in this group first excreted the challenge strain on Day 3 P.C. This organism was then isolated from all mice in this group almost everyday until the end of the observation period.

The organism was isolated from one mouse in the control group on the day following challenge infection. All mice in this group then excreted the organism continuously until the mice were euthanased (Table 37).

Autopsy:

The N-RM4 vaccinated mouse that died on Day 8 P.V. developed marked pathological changes, such as cloudy straw-colour fibrinous exudate in the thoracic and abdominal cavities, petechial haemorrhages of serosa of visceral organs, splenomegaly and grey-white foci scattered on the surface of the liver and the spleen. Three mice in the same group which survived throughout the observation period showed only mild to moderate pathological changes, such as splenomegaly. The other survivor had a few small grey-white foci on the surface of the kidney.

Four mice in the R-NM29 group euthanased due to clinical signs of salmonellosis developed splenomegaly and petechial haemorrhages of serosa of visceral organs. Two of these mice had marked fibrinous exudate in the abdominal cavity and small

grey-white foci scattered across the entire surface of the liver. The surviving mice in this group showed mild to moderate pathological changes, such as mild splenomegaly. One of them also had a few small grey-white foci on the surface of the liver.

Isolation of *Salmonella* from heart blood:

As indicated in Table 37, *Salmonella* was not isolated even using the EI method from heart blood of mice which survived throughout the observation period. In contrast, the challenge strain was isolated from all heart blood specimens from mice in R-NM29 group and the control group which were euthanased due to clinical signs of salmonellosis.

Quantitative and qualitative isolation of *Salmonella* from liver plus spleen:

A high number of *Salmonella* (6.5×10^8 cfu/g) was isolated from a liver plus spleen specimen of the mouse which died 8 days after vaccination with N-RM4. *Salmonella* was isolated only with the qualitative method from the organs of three of the four remaining mice in this group that survived throughout the observation period. Interestingly, only the vaccine strain was isolated from two of the three mice and the challenge strain was the only isolate from the remaining mouse (Tables 37 and 38 and Figure 28).

When R-NM29 was used as a vaccine, the challenge strain was isolated in numbers that could be quantified from the liver plus spleen specimens of four mice that were euthanased due to salmonellosis. The challenge strain was also isolated in numbers that could be quantified from two out of four mice in this group which survived throughout the observation period. However, the strain was only isolated following enrichment from the other two mice. Based on the results of quantitative measurements, the number of the organisms in the organs of the euthanased mice ranged between 1.1×10^9 and 2.4×10^9 cfu/g (with a mean of 1.8×10^9), as compared with between 0 and 7.2×10^5 cfu/g (with a mean of 1.8×10^5) in the mice that survived challenge infection (Tables 37 and 38 and Figure 28).

The challenge strain was isolated quantitatively from the organs of control mice. The numbers ranged between 5.3×10^8 and 3.3×10^9 cfu/g (with a mean of 1.7×10^9) - see Table 37 and Figure 28.

Isolation of *Salmonella* from organ samples:

Organ sample culturing was conducted for samples collected from 8 mice (4 from each vaccinated group) which survived throughout the observation period, as negative results for heart blood culturing were obtained from these mice. In the N-RM4 group, only the challenge strain was isolated from one out of four mice and the vaccine strain was the only isolate from the remaining mice. The organs from which *Salmonella* was isolated were the liver and spleen, ileocaecum and kidney (Table 38).

The challenge strain was the only isolate from organ samples of R-NM29 vaccinated mice. The organs from which the organism was isolated were the liver and spleen, ileocaecum, caecum, colon and kidney (Table 38).

TABLE 37 : *S. dublin* VACCINE TRIALS IN MICE USING N-RM4 OR R-NM29 FOLLOWED BY CHALLENGE INFECTION WITH WILD STRAIN FD436.

Group		1[4]	2	3 (control)
Number of surviving mice	Initial number of mice	5	8	5
	Pre-challenge	4	8	5
	Post-challenge	4	4	0
Protection (%)		100	50	0
Faecal *Salmonella* isolation[2]	Pre-challenge	80/143	0/272	0/170
	Post-challenge	22/72 (23/72)[5]	65/106	11/20
Heart blood culturing[3]		0/4	4/8	5/5
Isolation of *Salmonella* from liver plus spleen[3]		1/4 (2/4)[5]	8/8	5/5

[1]: Vaccination: Group 1 = N-RM4, 2 = R-NM29. Booster vaccination: on Day 21 P.V. (only for Group 2). Challenge infection: on Day 35 P.V. Refer to Table 33 for detailed vaccination and challenge infection information.

[2]: Number of cultures positive for the vaccine strain (pre-challenge) and the challenge strain (post-challenge) over the total number of samples tested in each group.

[3]: Number of cultures positive for the challenge strain over the number of samples tested at autopsy.

[4]: Results of protection, faecal *Salmonella* isolation, heart blood culturing and isolation of *Salmonella* from liver plus spleen do not include data obtained from the mouse which died before challenge infection.

[5]: Numbers in parentheses indicate the number of cultures positive for the vaccine strain over the total number of samples.

TABLE 38: DISTRIBUTION OF *Salmonella* IN INTESTINAL ORGANS OF MICE THAT SURVIVED THROUGHOUT VACCINE TRIALS USING N-RM4 OR R-NM29 FOLLOWED BY CHALLENGE INFECTION WITH FD436.

Group[1] / Organ	1 (n = 4)	2 (n = 4)
Lung	0/4[2]	0/4
Liver & spleen	1S, 2R/4	4S/4
Duodenum & jejunum	0/4	0/4
Ileum	0/4	0/4
Ileocaecum	1S, 1R/4	3S/4
Caecum	0/4	3S/4
Colon	0/4	2S/4
Kidney	1S, 2R/4	1S/4

[1]: Vaccination: Group 1 – N-RM4, 2 – R-NM29. Refer to Table 33 for detailed vaccination and challenge infection information.

[2]: Results represent the numbers of cultures positive for the corresponding vaccine strains and the challenge strain over the total number of samples tested in each group. S = nalidixic acid and rifampicin sensitive (challenge) strain, R = nalidixic acid and rifampicin resistant (vaccine) strains.

EXAMPLE 7

Vaccine trials in calves

<u>Experimental procedures for Example 7:</u>

Experimental animals:

For the first vaccine trial, eight 3 to 4 week old male calves (Calf No. 1 – 8) will be obtained from a herd known to be free of *S. dublin* infection and divided into 4 groups. The calves will be given colostrum immediately after birth. Prior to the trials, faeces and sera from each calf will be tested bacteriologically and serologically to confirm the calves are free from *S. dublin* infection. The same process will be followed for a further 8 male calves (Calf No. A – H) for the second vaccine trial.

Feeding:

Milk replacement formula will be given twice a day (8 a.m. and 3 p.m.).

Clinical monitoring:

Clinical condition of the calves will be monitored three times a day at 9 a.m., 4 p.m. and 8 p.m.

Vaccination and challenge infection:

In the first trial, calves in groups 2 and 3 will be orally vaccinated and then calves in groups 3 and 4 will be challenge exposed via the oral route 14 days after vaccination (see Tables 1 and 2). In the second trial, calves in groups 6 and 7 will be vaccinated and then calves in groups 7 and 8 will be challenge exposed in the same manner (see Tables 39 and 41).

TABLE 39 : VACCINATION AND CHALLENGE INFECTION FOR FIRST TRIAL

	Group	Number of animals	Vaccine strain	Dose (cfu) & route	Challenge strain	Challenge dose (cfu) & route
1st trial	1	2	N/A	-	N/A	-
	2	2	M25	$10^{11\#}$ PO	N/A	-
	3	2	M25	$10^{11\#}$ PO	FD436	$10^{9\#}$ PO
	4	2	N/A	-	FD436	$10^{9\#}$ PO
2nd trial	5	2	N/A	-	N/A	-
	6	2	M25	$10^{11\#}$ PO	N/A	-
	7	2	M 5	$10^{11\#}$ PO	FD436	$10^{9\#}$ PO
	8	2	N/A	-	FD436	$10^{9\#}$ PO

To ensure the viability of bacteria after passage through the stomach, the following chemical solution will be administered with the bacteria :
1 g $MgCO_3$, 1 g magnesium trisilicate, 1 g $NaHCO_3$, 20 ml distilled water.

Study design:

1. Clinical signs will be monitored for 8 weeks following vaccination.
2. Faeces, whole blood and serum will be taken for bacteriological, haematological, biochemical and serological examination (Tables 2 and 3).

3. The calves will be euthanised on Day 56 or upon showing clinical signs of salmonellosis and subject to full post-mortem examination including obtaining tissue specimens for bacteriological and histological examination.

Organs and contents (cont.) that will be bacteriologically investigated are as follows:

Lung, liver, spleen, kidney, abomasal cont., duodenal cont., jejunal cont., ileal cont., ileocaecal cont., caecal cont., colonic cont., ileal mesenteric lymph nodes (MLN), ileocaecal MLN, colonic MLN and bile.

Organs that will be histopathologically investigated are as follows:

Liver, spleen, gall bladder, kidney, duodenum, jejunum, ileum, ileocaecum, caecum, colon and mesenteric lymph nodes. Gall bladders will be further investigated through electron microscopy.

The vaccine trial will be repeated three times to obtain adequate numbers for statistical confidence.

Results for Example 7:

1. No calves produced clinical symptoms, except soft stools, post-vaccination using M25.
2. *Salmonella* was isolated from faeces of vaccinated calves using the direct isolation method for 1 – 2 days post-vaccination. The organism was not isolated from faeces beyond 8 days following vaccination even using the enrichment method.
3. Post lethal challenge infection, all vaccinated calves had a fever (39.5 – 40.5°C) and watery diarrhoea for several days, but they were relatively active and had a stable appetite. Compared with this, calves in the positive control group (non-vaccinated group) produced critical symptoms, such as very high fever (>40.5°C), depression, anorexia, tachypnea and tachycardia, but only a small amount of diarrhoea.

4. Numbers of *Salmonella* (a nalidixic acid and rifampicin sensitive strain that was suspected of being the challenge strain) excreted in the faeces of vaccinated calves following challenge infection were significantly ($p = 0.0242$) fewer than those in the faeces of calves in the positive control group.
5. Upon autopsy, *Salmonella* (the challenge strain) was isolated from various organs and tissues, including the lung, kidney, bile, bone marrow and cerebrospinal fluid, of calves in the positive control group. However, in the vaccinated and challenge group, the strain was isolated only from the intestine and mesenteric lymph node.
6. In the vaccinated and challenge group, no *Salmonella* was isolated from any organs tested 32 days post-vaccination (18 days post-challenge).

Current findings:

1. Single vaccination with M25 protected calves from lethal challenge infection.
2. The vaccine strain was excreted in the faeces only for a short period (≤8 days).
3. The vaccine eliminated the challenge bacteria from the host within 18 days.

TABLE 40 : *Salmonella* ISOLATION FROM FAECES AND BLOOD COLLECTED FROM CALVES IN VACCINE TRIALS

Calf	Sample	Day0	1	2	3	4	5	8	11	14	15	16	17	18	19	22	25	28	31	34	37	40
1	Faeces	-	-	-	-	-	-	-	-	-	-	-	Euthanased									
(NC)	Blood	-	-	-			-	-	-	-	-	-										
D	F	-	+DR	+ER	+ER	+ER	+ER	-	Euthanased													
(V)	B	-	-	-			+	-														
3	F	-	+DR	+ER	+ER	+ER	+ER	+ER	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(V)	B	-	+	-			-	-	-	-	-	-	-	-	-	-	-	-	-		-	-
4	F	-	+DR	+ER	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(V)	B	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	-		-	-
7	F	-	-	-	+E*	+E*	-	-	-	+E*	3x10^5	3x10^3	Dead									
(PC)	B	-	-	-			-	-	-	-	+	+										
8	F	-	-	-	-	-	-	-	-	-	2x10^7	5x10^4	Euthanased									
(PC)	B	-	-	-			-	-	-	-	+	+										
G	F	-	-	-	-	-	-	-	-	-	9x10^7	2x10^6	2x10^6	Euthanased								
(PC)	B	-	-	-			-	-	-	-	+	+	+									
H	F	-	-	-	-	-	-	-	-	-	7x10^7	7x10^7	8x10^7	Euthanased								
(PC)	B	-	-	-			-	-	-	-	-	-	+									
E	F	-	+DR	+ER	-	+ER	+ER	+ER	-	-	4x10^5	2x10^6	+E	Euthanased								
(VC)	B	-	-	-			+	-	-	-	-	-	-									
F	F	-	+DR	+ER	-	+ER	+ER	-	-	-	1x10^7	1x10^6	+E	Euthanased								
(VC)	B	-	-	-			-	+	-	-	-	-	-									
5	F	-	+DR	+ER	-	+ER	-	-	-	-	3x10^6	+E	+E	+E	-	-	-	-	-	Euthanased		
(VC)	B	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	-			
6	F	-	+DR	+DR	+ER	+ER	-	-	-	-	2x10^6	6x10^3	3x10^5	+E	+E	+E	-	-	-	Euthanased		
(VC)	B	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	-			
C	F	-	+DR	+ER	+ER	+ER	-	-	-	-	4x10^6	1x10^3	+E	+E	-	-	-	-	-	Euthanased		
(VC)	B	-	-	-			-	-	-	-	-	-	-	-	-	-	-	-	-			
2	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+E	-		Euthanased		
(NC)	B	-	-	-			-	-	-	-	-	-	-	-	+	+	+	+				
A	F	-	-	-	-	-	-	-	+E	-	-	4x10^3	Euthanased									
(NC)	B	-	-	-			-	-	+	+	+	+										
B	F	-	-	-	-	+E	-	+E	+E	+E	-	-	Euthanased									
(NC)	B	-	-	-			-	-	+	+	-	+										

Day0 = Vaccination; Day14 = Challenge infection; NC = Negative control; PC = Positive control; V = Vaccinated; VC = Vaccinated and challenged; + = positive; - = negative; D = direct isolation method; E = enrichment isolation method; R = Nalidixic acid & rifampicin resistant strain; * = *Salmonella* other than *S. dublin.*

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TABLE 41 : *Salmonella* ISOLATION FROM ORGAN, TISSUE AND CONTENT SAMPLES COLLECTED FROM CALVES IN VACCINE TRIALS

Group	Calf	Autopsy Date	Organ, tissue and content											
			D.C.	I.C.	C.C.	M.L.N.	Liver	Spleen	Bile	Kidney	Lung	A.S.	B.M.	C.S.F.
Negative control	1	Day16	-	-	-	-	-	-	-	-	-	-	-	-
	A	16	$5x10^6$	$5x10^6$	$7x10^6$	+	$2x10^6$	$2x10^6$	$3x10^7$	+	+	-	-	-
	B	16	+E	+E	+E	+	$1x10^5$	$4x10^4$	-	+	+	-	-	-
	2	30	-	+E	-	-	-	-	-	-	-	-	-	-
Positive control (Challenge only)	8	16	$7x10^5$	$3x10^7$	$1x10^7$	+	$1x10^4$	$2x10^5$	-	+	+	-	+	+
	7	17(Dead)				+	+	+	+	+	+	+	+	+
	G	18	$2x10^4$	$5x10^4$	$2x10^5$	+	$2x10^3$	$2x10^3$	-	+	-	-	-	-
	H	18	$1x10^8$	$2x10^7$	$4x10^6$	+	$9x10^4$	$1x10^4$	$5x10^7$	+	+	-	-	-
Vaccination	3	40	-	-	-	-	-	-	-	-	-	-	-	-
	4	40	-	-	-	-	-	-	-	-	-	-	-	-
Vaccination & challenge	D	8(Dyspnea)	-	$3x10^2$R	$1x10^2$R	+R	$6x10^2$R	$2x10^2$ R	-	-	Pasteurella	-	-	-
	E	18	$5x10^4$	$8x10^6$	$2x10^6$	+	-	-	-	-	-	-	-	-
	F	18	$8x10^6$	$3x10^6$	$2x10^5$	+	-	-	-	-	-	-	-	-
	5	32	-	-	-	-	-	-	-	-	-	-	-	-
	6	32	-	-	-	-	-	-	-	-	-	-	-	-
	C	32	-	-	-	-	-	-	-	-	-	-	-	-

Day0: Vaccination - 10^{10} cfu/calf P.O.; Day14: Challenge infection – 10^9 cfu/calf P.O. M.L.N. = Mesenteric lymph node; A.S. = Abdominal swab; B.M. = Bone marrow; C.S.F. = Cerebrospinal fluid; D.C. = Duodenal contents; I.C. = Ileal contents; C.C. = Colic contents; E = *Salmonella* were isolated only using the enrichment method; + = Positive for *Salmonella* isolation; - = Negative for *Salmonella* isolation; R = Nalidixic acid and rifampicin resistant strain.

5

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DATED this 12th day of February, 2004

The University of Queensland

by its Patent Attorneys
DAVIES COLLISON CAVE

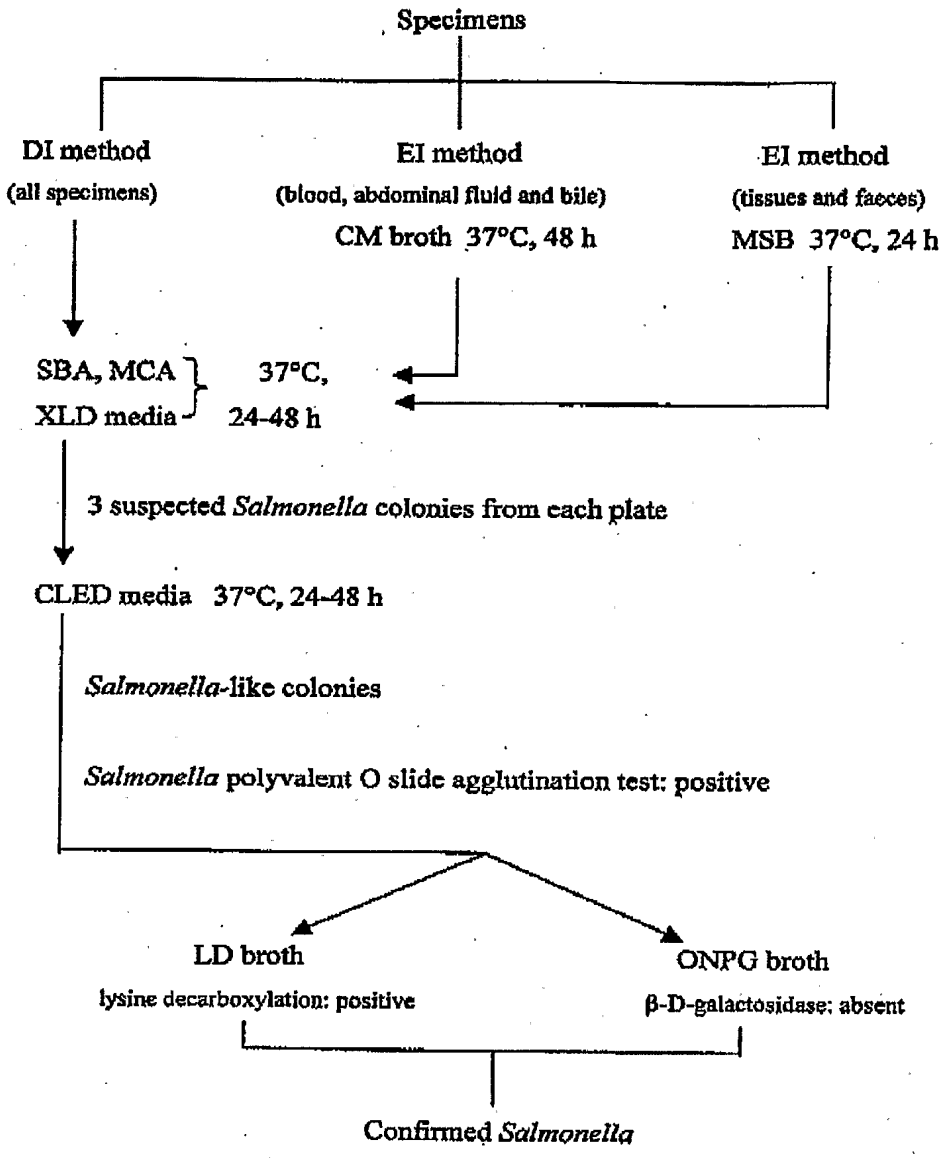

Specimens
DI method
(all specimens)
EI method
(blood, abdominal fluid and bile)
CM broth 37°C, 48 h
EI method
(tissues and faeces)
MSB 37°C, 24 h
SBA, MCA
XLD media
37°C,
24-48 h
3 suspected Salmonella colonies from each plate
CLED media 37°C, 24-48 h
Salmonella-like colonies
Salmonella polyvalent O slide agglutination test: positive
LD broth
lysine decarboxylation: positive
ONPG broth
β-D-galactosidase: absent
Confirmed Salmonella

FIGURE 1

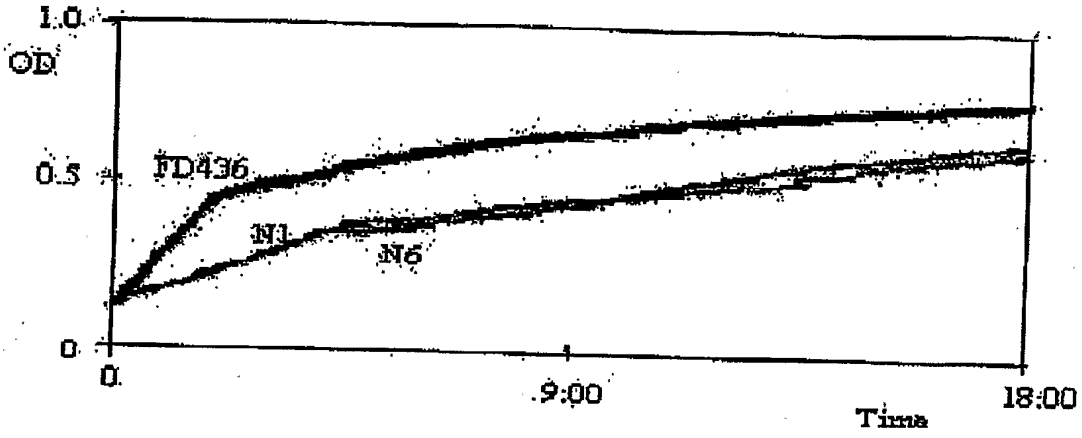


FIGURE 2

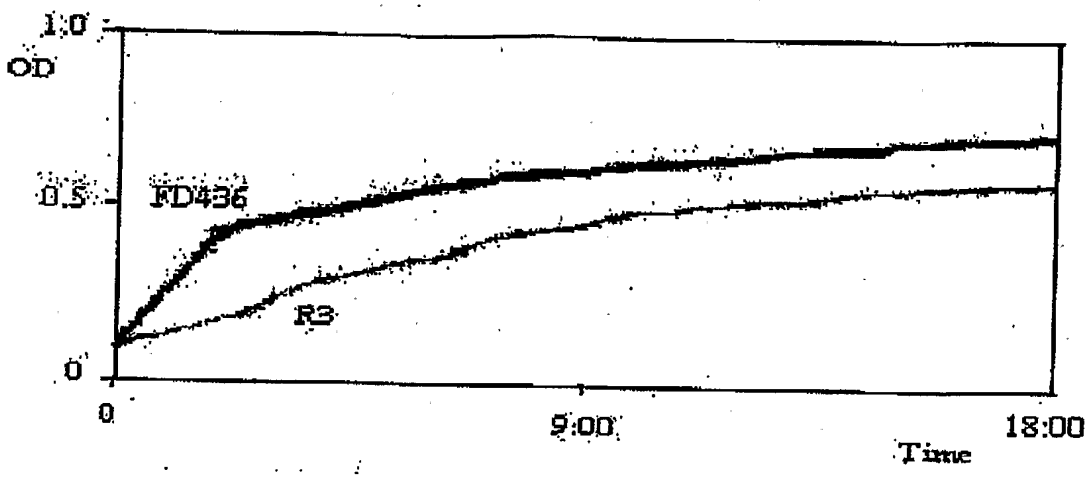


FIGURE 3

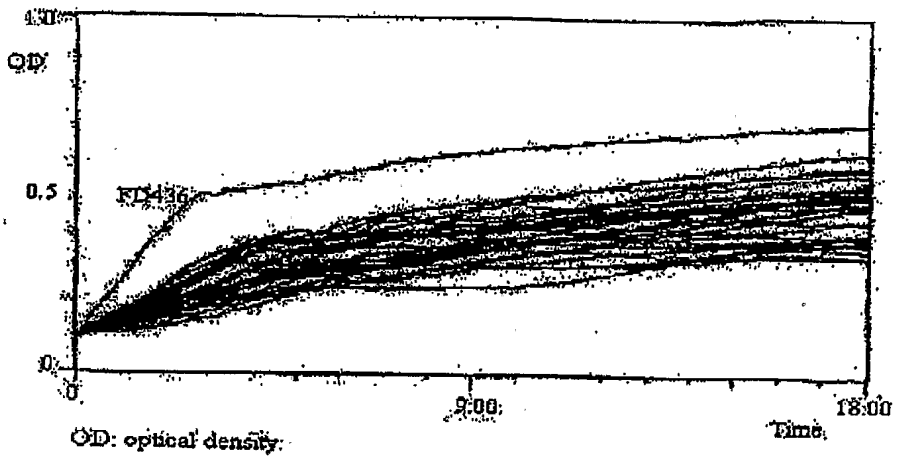


FIGURE 4

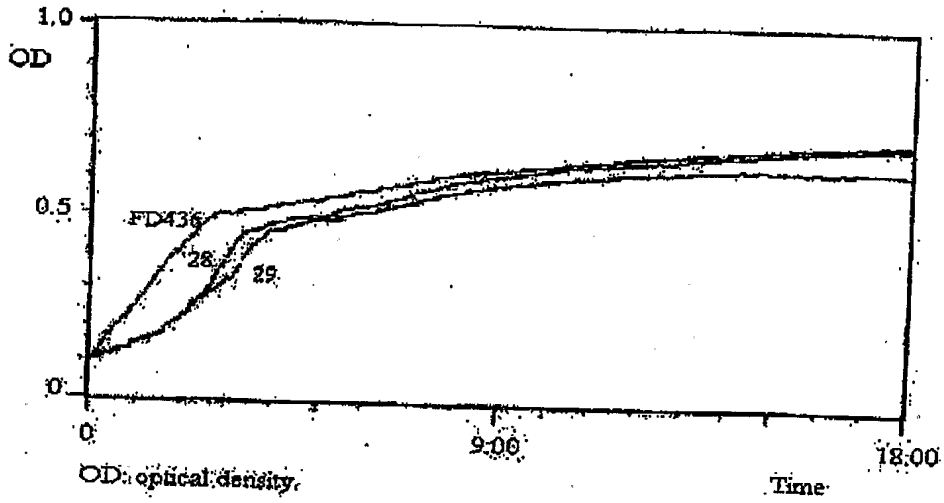


FIGURE 5

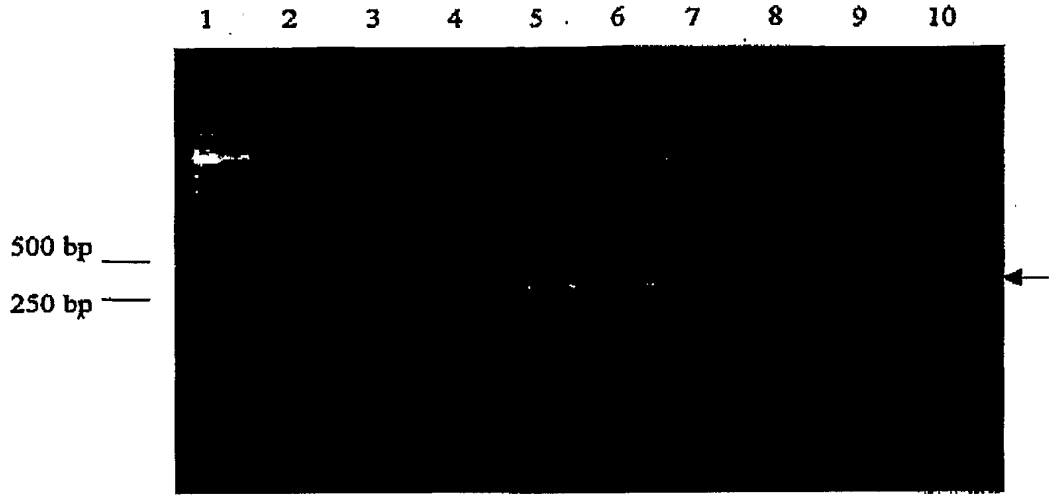


FIGURE 6

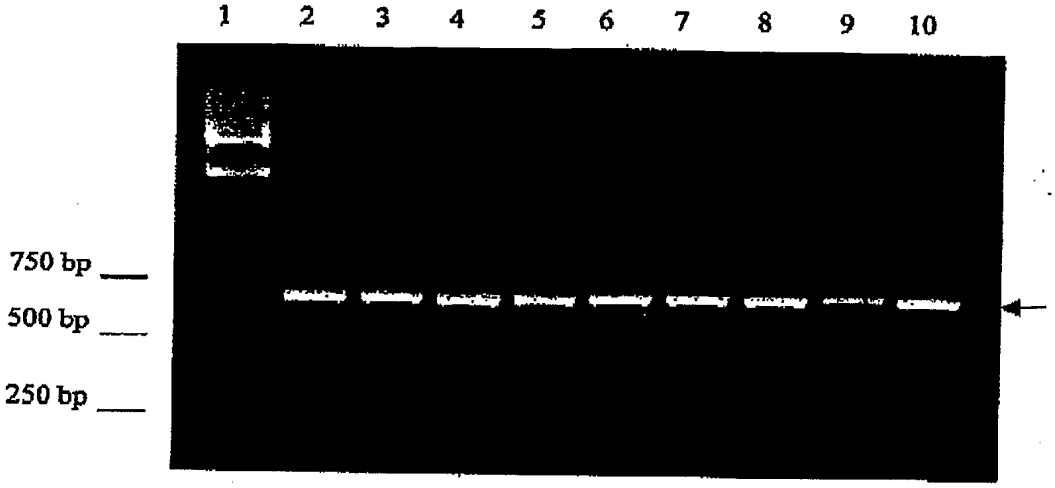


FIGURE 7

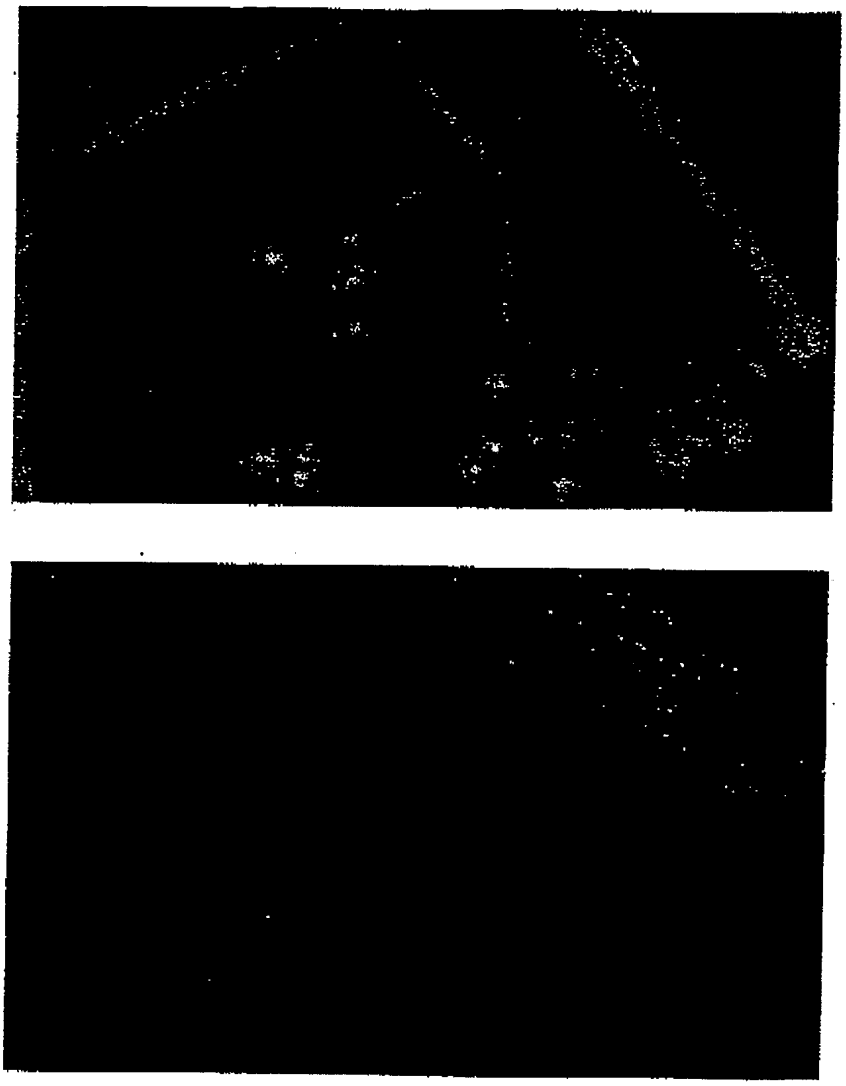


FIGURE 8

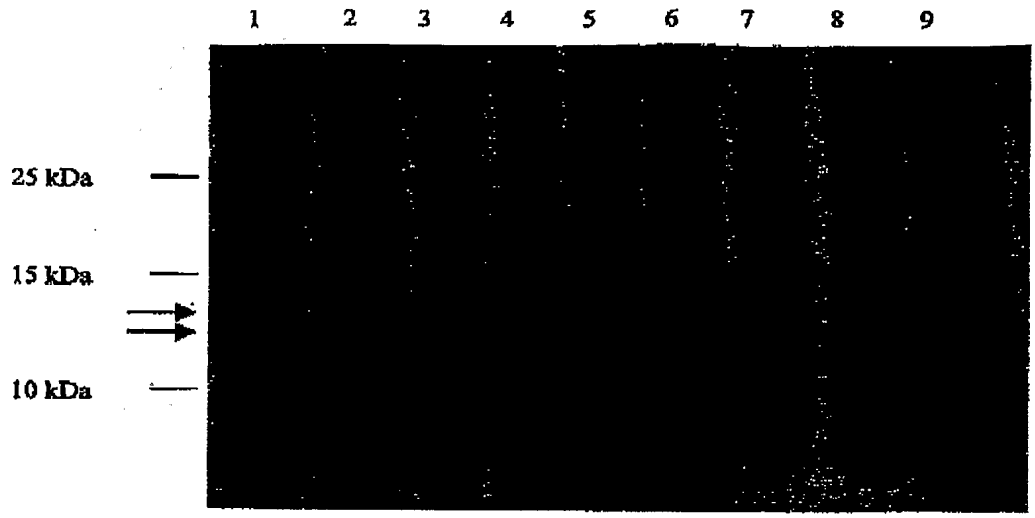


FIGURE 9

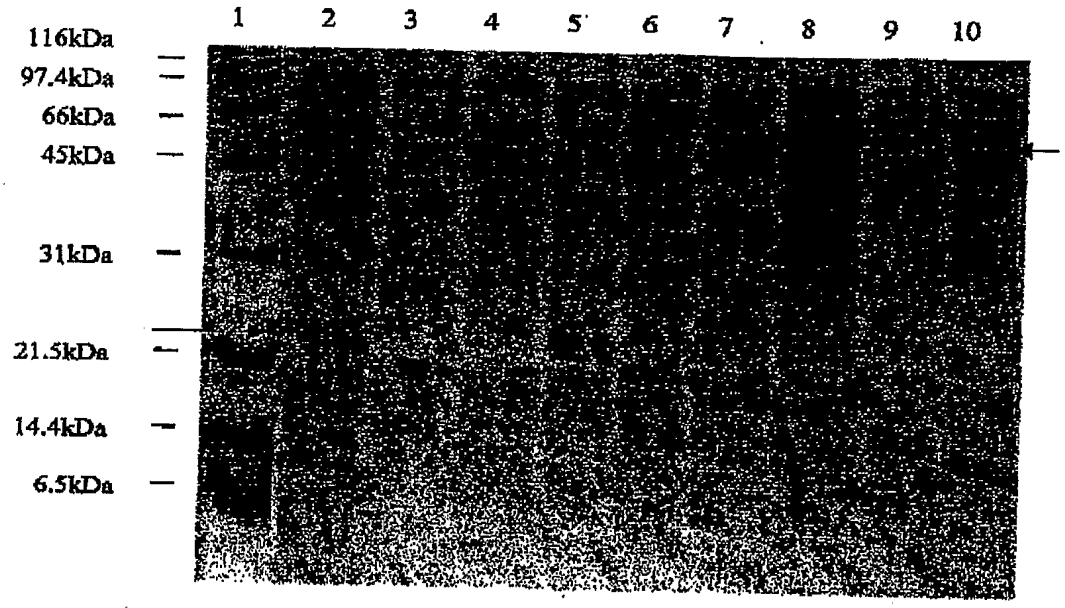
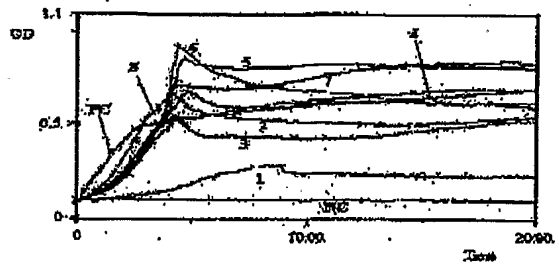
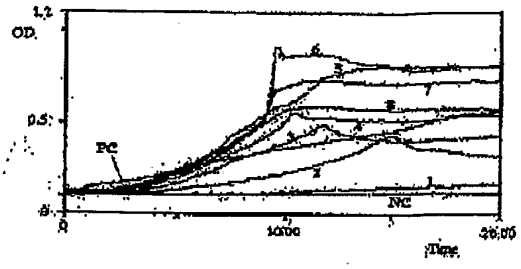

1
2
3
4
5
6
7
8
9
10
116kDa
97.4kDa
66kDa
45kDa
31kDa
21.5kDa
14.4kDa
6.5kDa

FIGURE 10

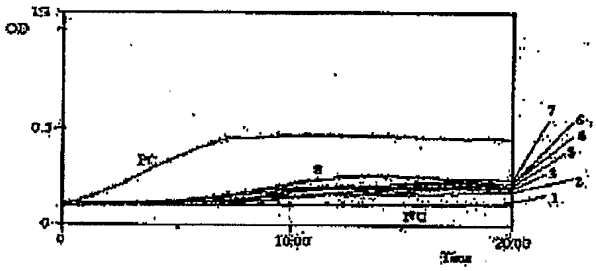
A – FD436



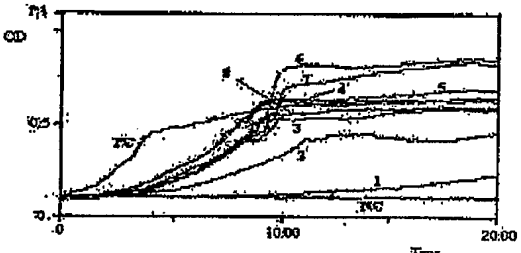
B – N-RM4



C – N-RM25

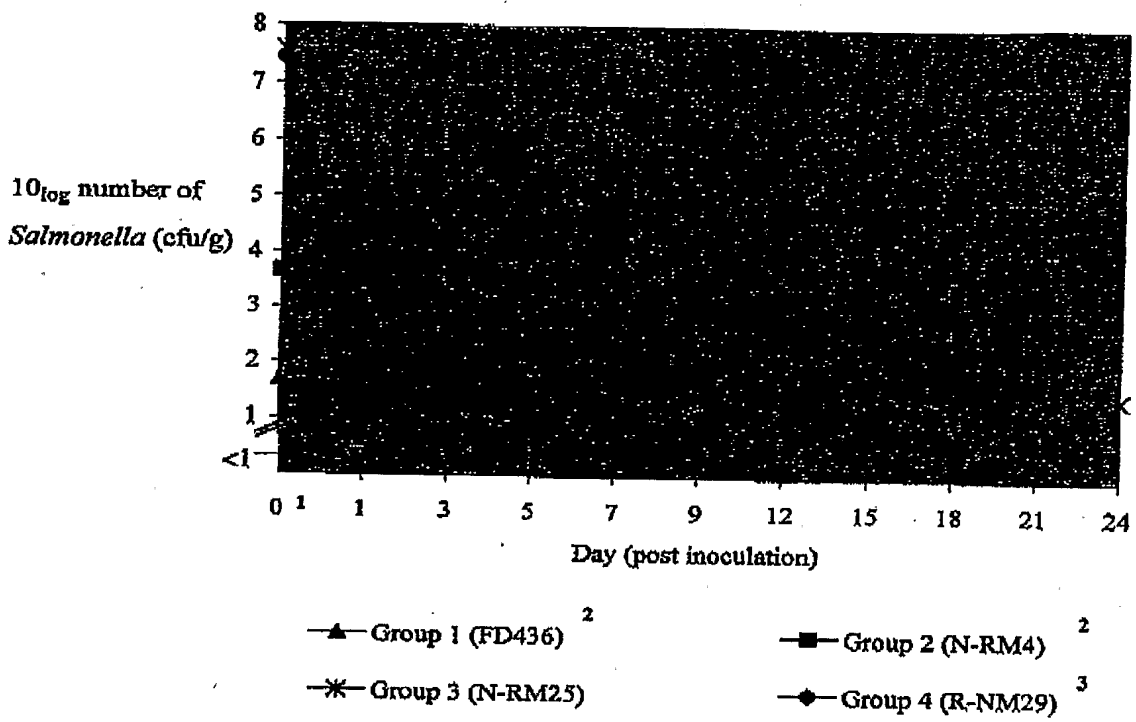


D – R-NM29



Concentration of bile salts No. 1: Figures in parentheses are estimated corresponding concentrations of crude bile salts.
1: 5.0% (28.85%), 2: 4.5% (14.45%), 3: 2.4% (7.2%), 4: 1.25% (3.6%), 5: 0.6% (1.8%), 6: 0.3% (0.9%), 7: 0.15% (0.45%), 8: 0.075% (0.225%).
PC: positive control, NC: negative control, OD: optical density.

FIGURE 11



[1]: The numbers of *Salmonella* on Day 0 indicate the infectious doses of FD436, N-RM4, N-RM25 and R-NM29 inoculated to mice in each group. They do not represent the number of bacteria in the liver plus spleen.
[2]: All mice in Groups 1 and 2 were euthanased or died by Day 6 and Day 8 post-inoculation, respectively.
[3]: No *Salmonella* were isolated from the livers and spleens of mice in Group 4 throughout the nine-day observation period.

FIGURE 12

FIGURE 13

FIGURE 14

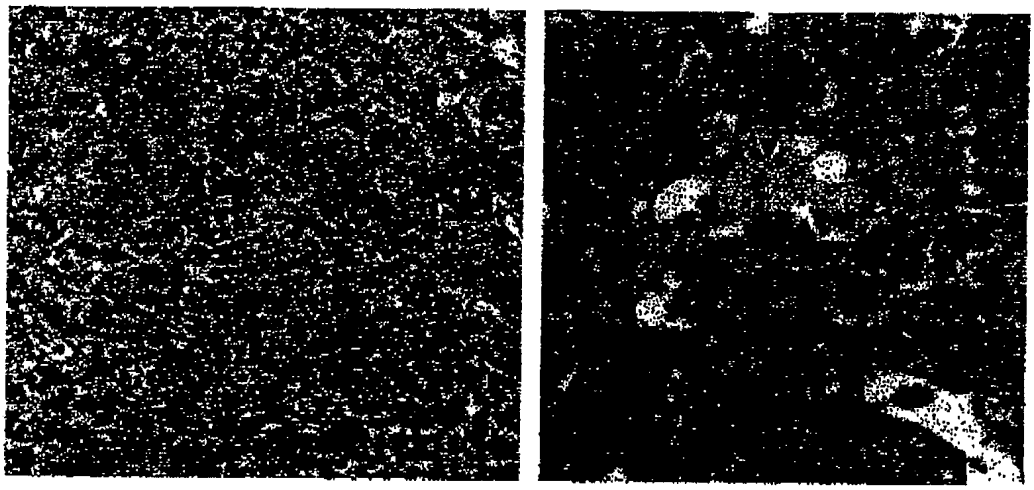


FIGURE 15

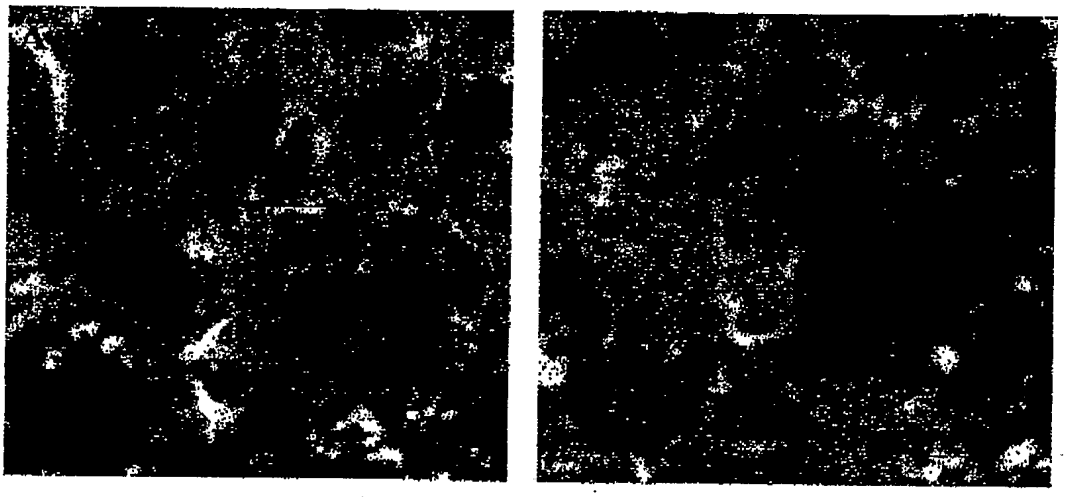


FIGURE 16

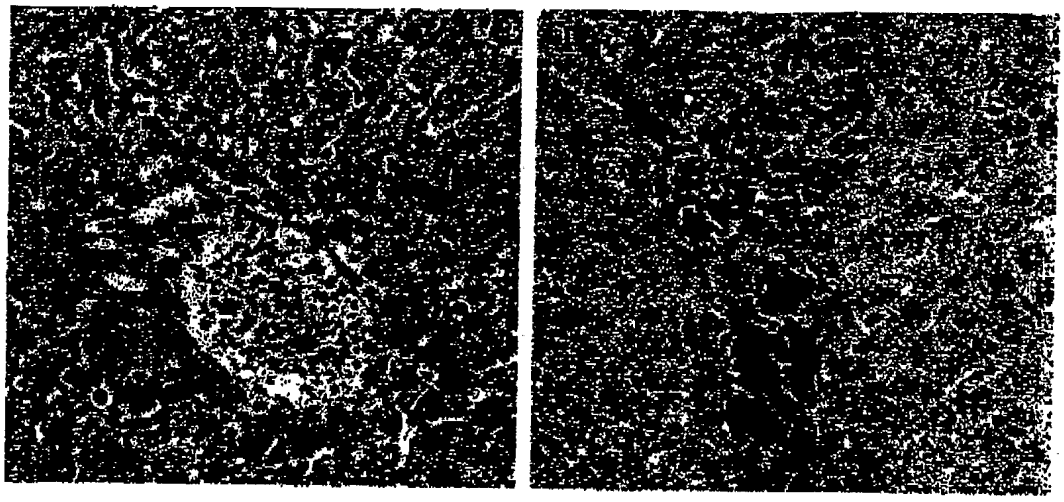


FIGURE 17

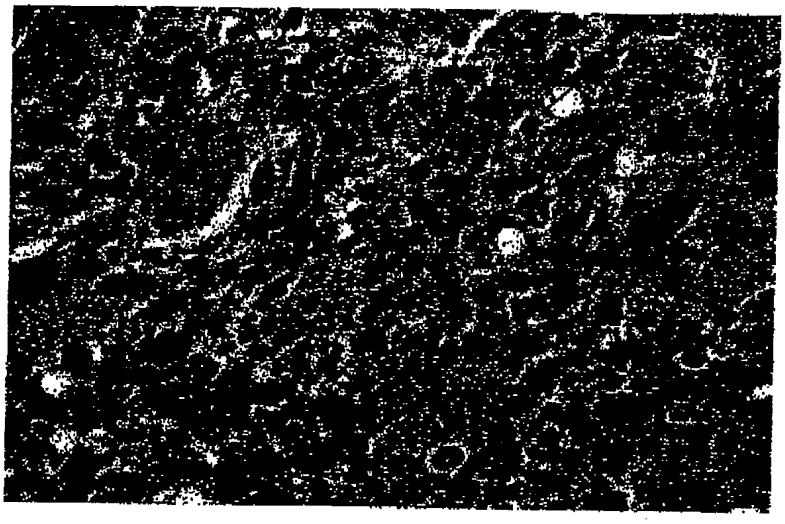


FIGURE 18

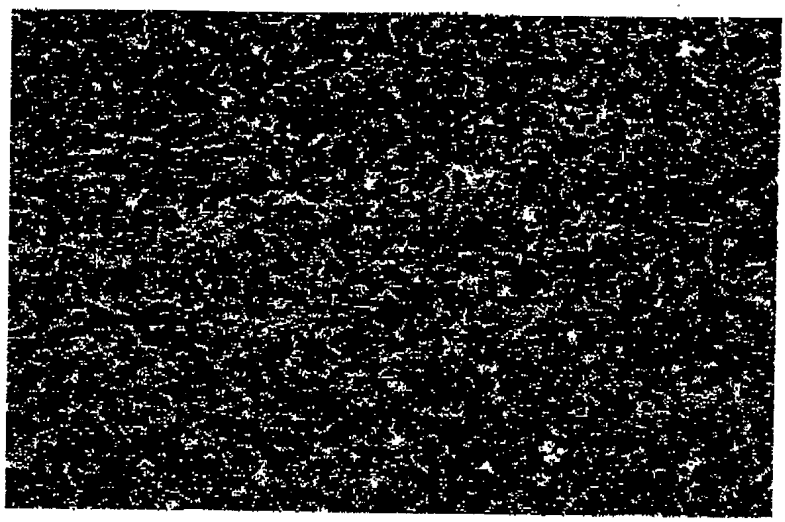


FIGURE 19

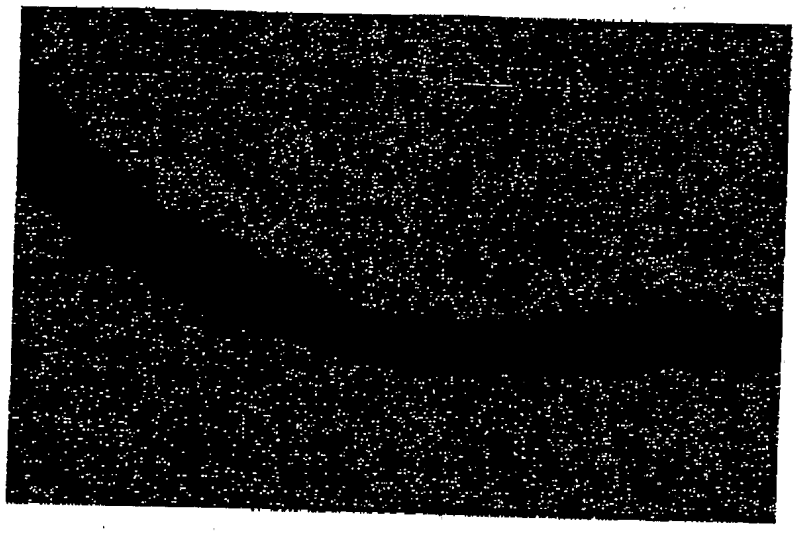


FIGURE 20

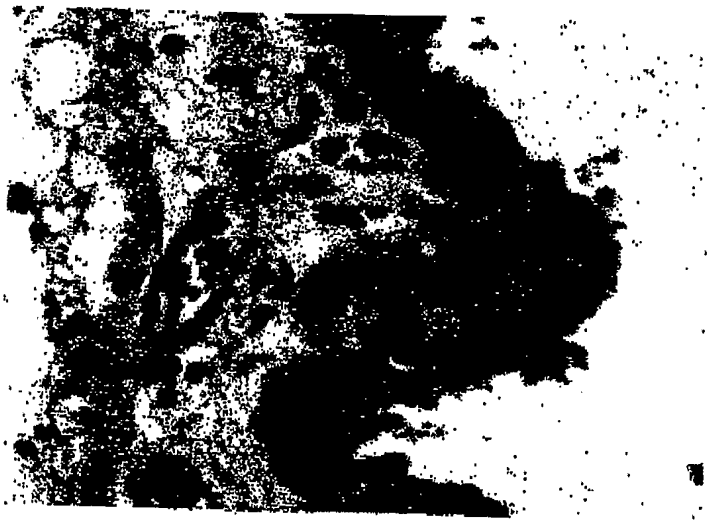


FIGURE 21

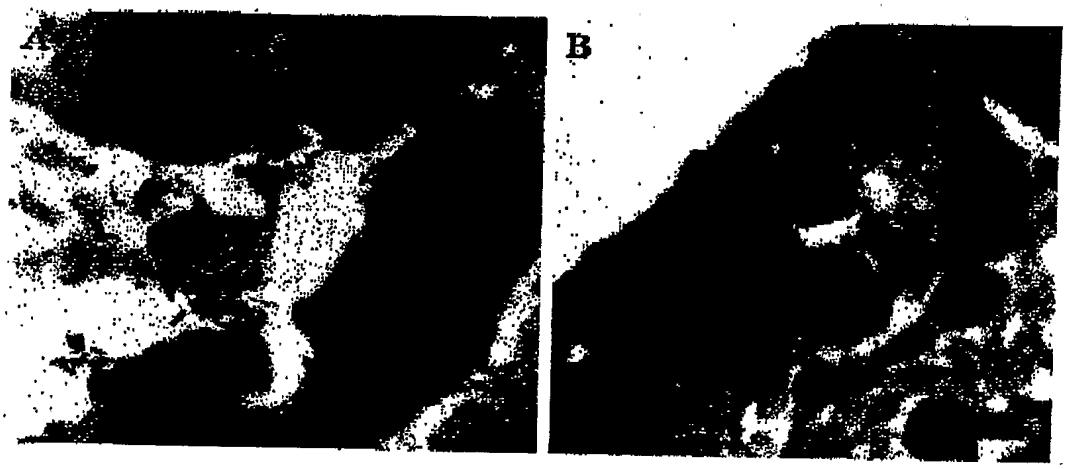


FIGURE 22

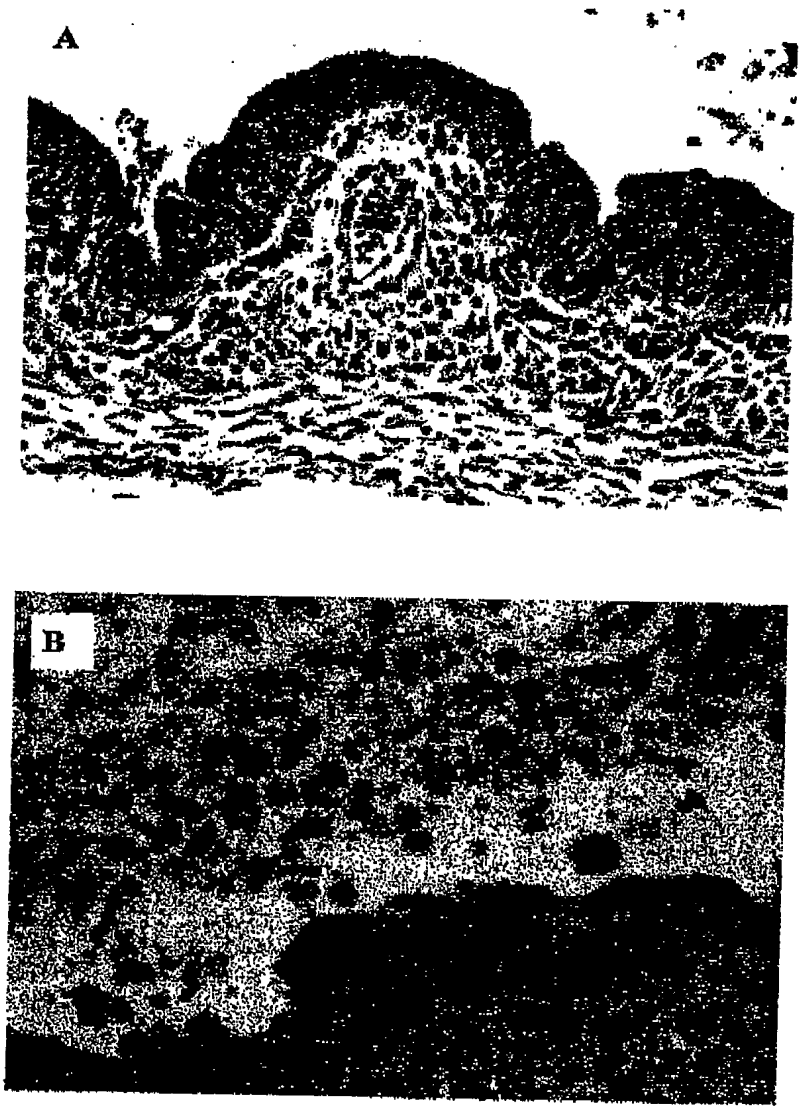


FIGURE 23

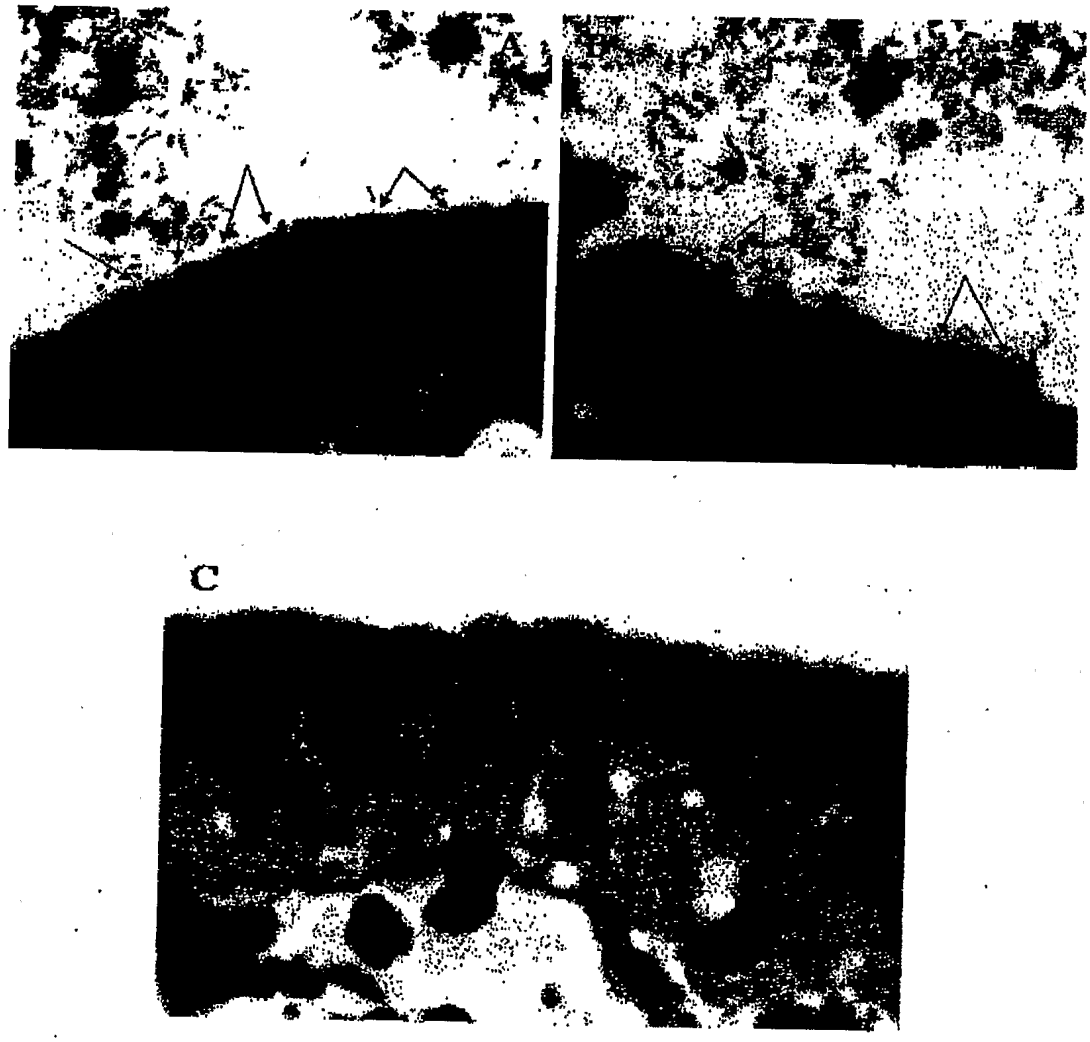


FIGURE 24

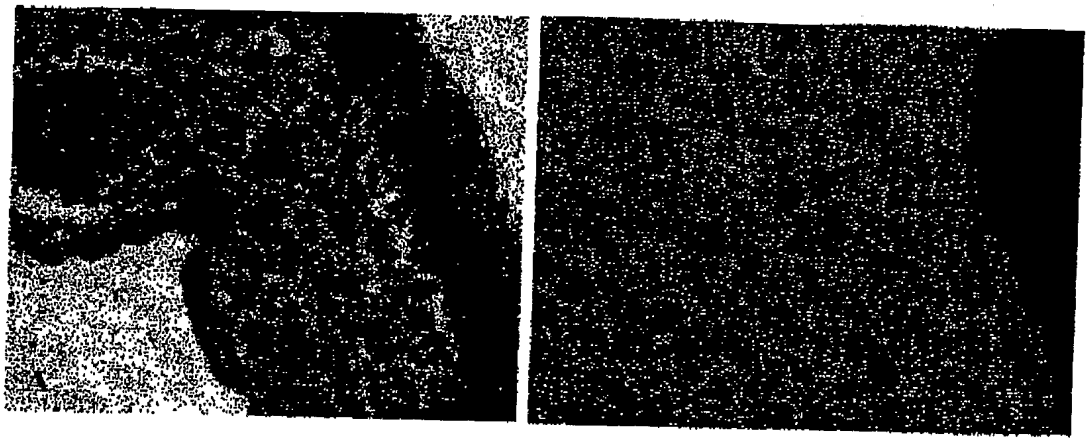


FIGURE 25

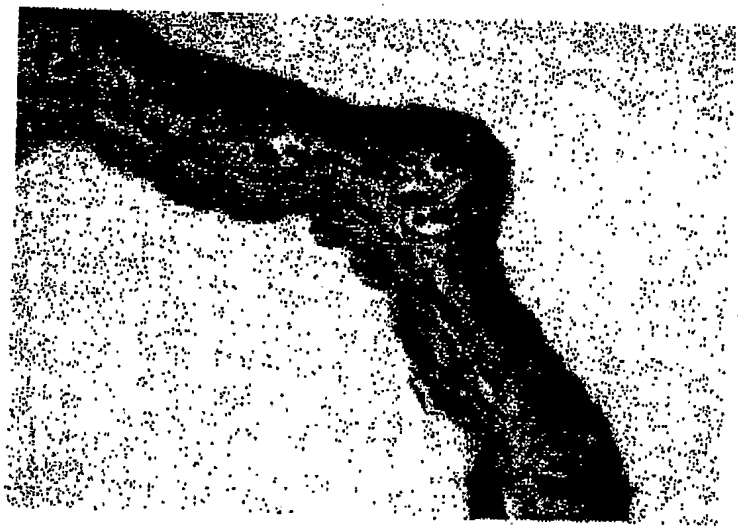
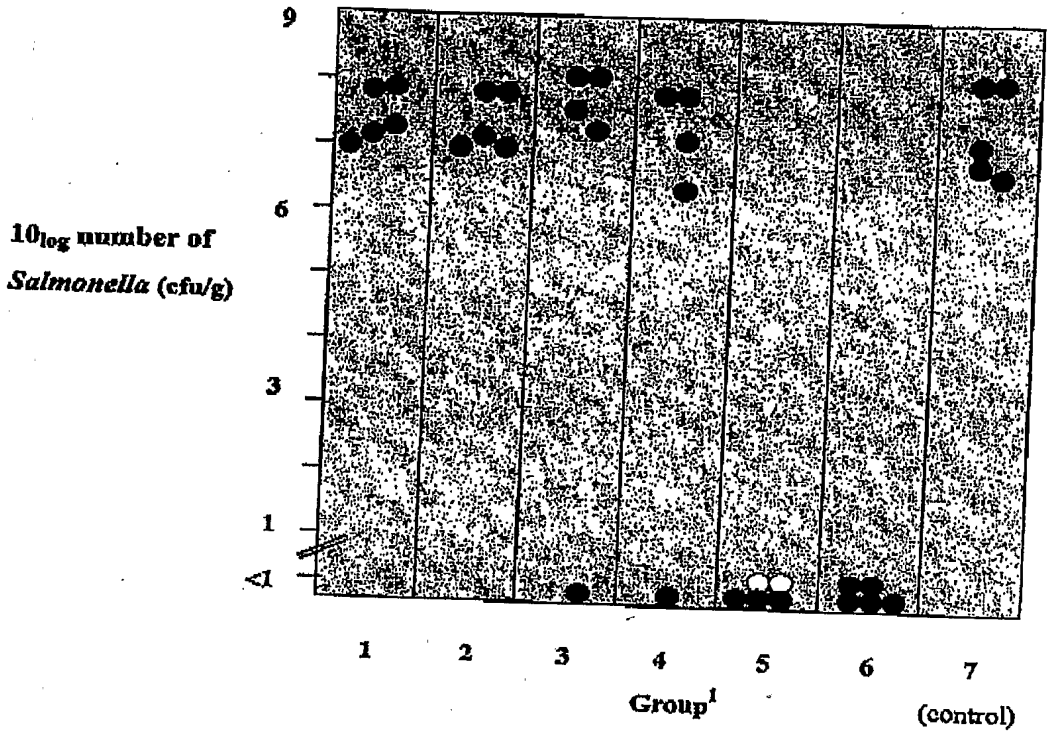


FIGURE 26



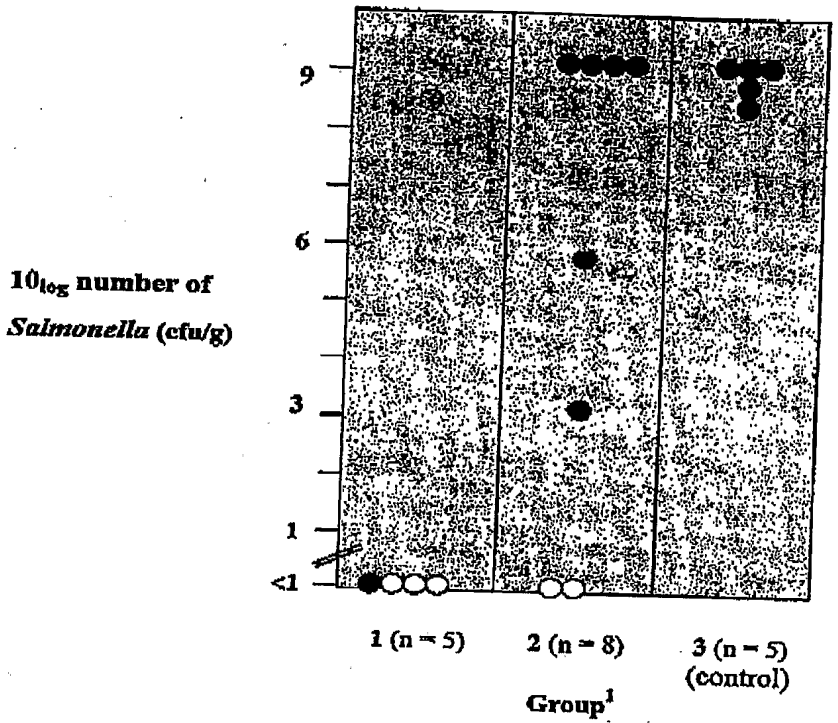
[1]: Vaccination: Group 1 – single low-dose, 2 – double low-dose, 3 – single medium-dose, 4 – double medium-dose, 5 – single high-dose, 6 – double high-dose. Refer to Table 8.2 for detailed vaccination and challenge infection information.

● : Mice euthanased due to clinical signs of salmonellosis and from which the challenge strain was isolated using quantitative culture.

○ : Mice that survived challenge infection and from which the challenge strain was isolated by enrichment only.

● : Mice that survived challenge infection and from which *Salmonella* was not isolated.

FIGURE 27



[1]: Vaccination: Group 1 - N-RM4, 2 - R-NM29. Refer to Table 8.3 for detailed vaccination and challenge infection information. n: number of tested mice.

- ◍: Mice that died before challenge infection and from which the corresponding vaccine strains were isolated using quantitative culture.
- ○: Mice that survived challenge infection and from which the corresponding vaccine strains were isolated by enrichment only.
- ●: Mice euthanased due to critical illness and from which the challenge strain was isolated using quantitative culture.
- ●: Mice that survived challenge infection and from which the challenge strain was isolated using quantitative culture.
- ○: Mice that survived challenge infection and from which the challenge strain was isolated by enrichment only.
- ●: Mice that survived challenge infection and from which *Salmonella* was not isolated.

FIGURE 28